The Inhibition of High and Low Molecular Weight Urokinase in Plasma

By Genesio Murano, David Aronson, Lynne Williams, and Larry Brown

The rates of inhibition of high molecular weight (HMW) and low molecular weight (LMW) urokinase (UK) incubated in plasma or with purified antithrombin III (AT-III) were compared. Using a fibrinolytic assay system to determine residual biologic activity, polyacrylamide gel electrophoresis to demonstrate the formation of complexes, and selective immunoprecipitation techniques to identify the plasma inhibitors participating in the neutralization process, it was established that: (A) HMW-UK is inhibited more rapidly than LMW-UK, both in plasma and with purified AT-III; (B) heparin (3–10 U/ml) accelerates the neutralization process in both systems, but only slightly; and (C) in plasma, several inhibitors, α2-macroglobulin, α1-antitrypsin, and antithrombin III, neutralize the activity of HMW-UK and LMW-UK.

UROKINASE, a serine protease produced in the kidney and found in urine, is a potent activator of plasminogen. Two major molecular forms are found in current therapeutic preparations: the high molecular weight (HMW) and low molecular weight (LMW) forms (55,000 and 35,000, respectively). The LMW species is derived from the HMW species by proteolysis. Immunologically, the two forms are similar. Depending on the method of preparation, either one or the other (or in varying proportions) are isolated. Products isolated from urine or from kidney tissue cultures are equally effective in inducing a fibrinolytic state in man.

With the observations that human plasma inhibits the activity of urokinase, that purified α2-macroglobulin, α1-antitrypsin, and antithrombin III form complexes with urokinase, and with the growing interest in the treatment of thromboembolic disease with this enzyme, it has become imperative to study the two molecular forms not only in terms of their plasminogen activation potential and in vivo clearance rates, but also in terms of their disposition with respect to interactions with naturally occurring protease inhibitors. In this study, we report on the rate of inhibition of HMW-UK and LMW-UK and present evidence in support of the inhibitory role of α2-macroglobulin, antithrombin III, and α1-antitrypsin in human plasma.

MATERIALS AND METHODS

All reagents employed in these studies were analytical grade.

Urokinase (UK)

HMW-UK (Winkinase, Sterling Winthrop Laboratories) and LMW-UK (Abbokinase, Abbott Laboratories) were purified by affinity chromatography on benzamidine-Sepharose according to Biologics FDA. 8800 Rockville Pike, Bethesda, Md. 20205. Amicon Co., desalted on Sephadex G-25 (Pharmacia) in 0.1 M ammonium acetate, pH 4.2, and lyophilized. The specific activity (SA) of these products was 124,000 U/mg for LMW-UK and 152,000 U/mg for HMW-UK. Based on quantitative gel scans, LMW-UK was greater than 96% pure, whereas HMW contained ~13% LMW-UK and ~3% unidentified very high molecular weight material.

125I-Urokinase (125I-UK).

Purified HMW-UK and LMW-UK (0.5 mg each) were independently adsorbed to benzamidine-Sepharose (column 1.5 x 0.9 cm) according to Holmberg et al. Iodination was performed in situ by percolating 1 ml of chloramine-T (0.2 mg/ml) containing 10 μl of 125INa (SA, 50 mCi/ml). The column was washed with 10 ml of equilibrating buffer (0.1 M sodium phosphate, 0.4 M sodium chloride, pH 7.0). The iodinated product was eluted with 5 ml of 0.1 M sodium acetate, 0.4 M sodium chloride, 0.1% bovine serum albumin, pH 4.1, and dialyzed overnight at 4°C against the equilibrating buffer. Aliquots of the product were stored frozen at −70°C. The SA of the labeled products was 36,000 U/mg for LMW-UK and 31,000 U/mg for HMW-UK. It is noted that a considerable loss in activity occurred during iodination.

Antithrombin III (AT-III)

This inhibitor was isolated from human plasma by affinity chromatography according to Miller-Anderson et al., as modified by Williams. Biologic (heparin cofactor) activity was determined according to Bick et al.

Normal Human Plasma (NHP)

Blood was collected in 3.8% sodium citrate (1:10, v/v), formed elements removed by centrifugation, and the plasma stored at −70°C. A pool of 8 donors served as the standard NHP.

AT-III-Depleted Plasma

Human plasma depleted of AT-III was prepared by immunoadsorption. The gamma globulin fraction from 20 ml of rabbit antisera (anti-AT-III) was coupled to 100 ml (packed volume) of Agarose A, 15 M, according to Cuatrecasas. About 50 ml of human plasma was mixed with the immunoadsorbent for 90 min at 4°C and the plasma separated on a sintered glass filter. The plasma was assayed for AT-III biologic activity (none detected) and for immunoreactivity (less than 10% detected) by radial immunodiffusion (Behring). It was stored at −70°C.

Plasminogen-Depleted Plasma

Human plasma depleted of plasminogen was prepared by affinity chromatography on lysine-Sepharose. The product was stored at −70°C.


Chromatographic Procedures

Gel permeation chromatography was performed on Sephacryl-200 (Pharmacia) at room temperature. Samples (1 ml) were applied to a 1.6 x 95 cm column and developed in 0.02 M Tris (hydroxymethyl) aminomethane, 0.15 M NaCl, pH 7.3, at a flow rate of 6 ml/hr. Effluents were monitored at 280 nm with a Uvicord-II (LKB Instruments). The column was calibrated with ribonuclease A, ovalbumin, bovine serum albumin, aldolase, and ferritin (Pharmacia Fine Chemicals).

Electrophoresis

Analytical polyacrylamide gel electrophoresis (% and 7.5%) in the presence of 0.1% sodium dodecylsulfate was performed according to Weber and Osborn19 in nonreducing conditions. Calibration for molecular size was done using the same standards as described for gel permeation chromatography. Molecular size estimation of complexes formed during incubation was based on interpolation from the standard curve (relative mobility versus log MW).

Radioisotope Measurements

The radioactivity content of all products—purified 125I-urokinase, chromatography effluents, and gel slices—was determined with a gamma counter (Beckman Instruments).

Antisera

Antibodies to HMW-UK, LMW-UK, and AT-III were developed in goats by repeated intradermal injection of antigen in complete Freund's adjuvant. The serum was harvested and stored at -70°C. Antisera to a2-macroglobulin and a,-antitrypsin were obtained from Behring Diagnostics. Antiserum to a2-antiplasmin was kindly donated by Dr. D. Collen (University of Leuven, Belgium).

Assay for Urokinase

Principle. Both HMW-UK and LMW-UK were assayed by two methods: A and B. Method A was similar to that of Ploug and Kjeldgaard.20 Method B (courtesy of Dr. C. Schmakel, Abbott Laboratories) consisted of a slight modification of method A. Basically, both methods consisted of the formation of a fibrin clot containing plasminogen and UK. A plastic sphere was placed on top of the clot. The time required for the sphere to travel to the bottom was recorded as the “lysis time.”

Incubation of Urokinase With Purified AT-III

HMW-UK and LMW-UK were incubated at 37°C with purified AT-III at an equimolar ratio in 0.1 M sodium phosphate buffer, pH 7.4, with and without heparin (3 U/ml). At specified time intervals, appropriately diluted aliquots were assayed for residual activity and electrophoresed on 7.5% polyacrylamide gels containing 0.1% sodium dodecylsulfate (SDS). For the electrophoresis, the reaction was terminated by mixing 50 µl aliquots with 50 µl of SDS containing buffer and freezing at -70°C.

Incubation of Urokinase in Plasma

HMW-UK and LMW-UK were incubated at 37°C at concentrations of 25, 50, 100, 200, 1000, 2000, and 25,000 U/ml in normal plasma, in plasminogen-depleted plasma, in AT-III-depleted plasma, with and without heparin (3 U/ml) and in heparin alone (3-10 U/ml). At specified time intervals, appropriately diluted aliquots were assayed for residual activity and either electrophoresed on 5% polyacrylamide gels in the presence of 0.1% SDS or chromatographed on Sephacryl (the reaction terminated by the addition of 5 x 10^-3 M disopropylfluorophosphate). The study with plasminogen-depleted plasma was performed by adding HMW-UK to the plasma at a concentration of 25 U/ml. At the end of 60 min and again at the end of 120 min, an additional 25 U/ml were added. The disappearance of UK activity was monitored for each 60-min incubation period. For the experiments involving electrophoresis on polyacrylamide gels and chromatography on Sephacryl, 125I-HMW-UK and 125I-LMW-UK were used as tracers. In order to establish the distribution of urokinase upon incubation, gels were sliced (1-mm thickness) and counted in a gamma counter; fractions from the Sephacryl column representing inhibitor(s)–UK complexes were also counted in a gamma counter.

Identification of Urokinase–Inhibitor Complexes in Plasma

Aliquots (100 µl) of peak radioactive fractions from Sephacryl chromatograms were mixed with 100 µl each of anti-a2-macroglobulin, anti-a1-antitrypsin, anti-a2-antiplasmin, and anti-AT-III. Samples were incubated at 37°C for 1 hr, followed by 16 hr at 4°C. Samples were first counted in a gamma counter to obtain a total count, then centrifuged and the supernatant counted for residual radioactivity. The net difference was expressed as percent precipitable radioactivity.

RESULTS

A standard curve (assay method B) for urokinase activity at various concentrations is illustrated in Fig. 1. Similar profiles were obtained with assay method A, however, higher concentrations of urokinase had to be used in order to obtain similar lysis times. Values for residual activity in the various incubation mixtures were obtained by interpolating appropriately diluted samples from either standard curve.

The pattern of inhibition of HMW-UK and LMW-UK in normal human plasma (at 25, 50, 100, 1000,
Residual activity is expressed as percent of starting activity. At all concentrations, LMW-UK was inhibited more slowly and, particularly at very low concentrations, the loss of activity for both enzymes was apparently precipitous.

Figure 4 illustrates the inhibition of HMW-UK in normal and in plasminogen-depleted plasma. This experiment was performed in order to determine whether the precipitous loss of activity observed at low (therapeutic) UK concentrations (25-50 U/ml) (Fig. 2) was real, or whether the values obtained reflected the presence of (or generation of) influencing substances in the incubation mixture and carried over in significant quantities (due to a low dilution factor) into the assay mixture. At an incremental UK concentration of 25 U/ml (added at hourly intervals), it was noted that, in plasminogen-depleted plasma, at 0 time, at 60 min, and again at 120 min, a value of less than 40% of the original activity was found. This was also noted in normal plasma, but only upon the addition of the first 25 U/ml. With subsequent addition at 60 and 120 min, the apparently precipitous loss disappeared, indicating that upon the addition of UK, the plasminogen-depleted plasma did not change with respect to substances that influenced the assay system, whereas normal plasma did (see Discussion).

Figure 5 illustrates the rate of inhibition of HMW-UK (2000 U/ml) in normal plasma and in plasma almost depleted of AT-III, in the presence and absence of heparin (3 U/ml). Removal of the majority of the AT-III from plasma reduced, but did not abolish, the inactivation of HMW-UK, indicating the probable role of other inhibitors in the inactivation of UK. A similar profile (not illustrated) with slightly reduced rates of inhibition, was obtained with LMW-UK. It is noted that heparin, at 3 U/ml, accelerates the rate of inhibition slightly in both normal plasma and in AT-III-depleted plasma. Not illustrated are studies showing that, in normal plasma, at a heparin concentration of less than 1 U/ml, this effect was negligible; at a
Concentration higher than 3 U/ml, up to 10 U/ml, the effect became incrementally slightly more pronounced; at yet higher heparin concentrations (up to 15 U/ml), no further acceleration was noted. When incubated for 3 hr at 37°C in heparin alone (3–10 U/ml), both HMW-UK nor LMW-UK showed less than 10% loss of activity.

The inhibition of HMW-UK and LMW-UK activity with stoichiometric quantities of purified AT-III, with and without heparin, is illustrated in Fig. 6. The inhibition was about the same as that observed in plasma, with heparin slightly enhancing the reaction and HMW-UK being preferentially inactivated. The formation of enzyme–inhibitor complexes (UK–AT-III) is illustrated by the electrophoretic profiles in Fig. 7. The top quadrant of the figure illustrates the time-dependent formation of a high molecular weight complex when purified LMW-UK (first gel) and puri

Fig. 5. Inhibition of HMW urokinase (2000 U/ml) in heparin (3–10 U/ml) in normal and in AT-III-depleted plasma, with and without heparin at 3 U/ml (% residual activity versus incubation time at 37°C).

Fig. 6. Inhibition of LMW and HMW urokinase with equimolar quantities of purified AT-III, in the presence and absence of heparin at 3 U/ml (% residual activity versus incubation time at 37°C).

Fig. 7. Polyacrylamide gel (7.5%) electrophoretic profiles (in the presence of 0.1% sodium dodecylsulfate) of purified AT-III (second gel from left, top and bottom quadrant), purified LMW urokinase (first gel from left, top quadrant), purified HMW urokinase (first gel from left, bottom quadrant), and variously timed mixture incubates, from 0 to 3 hr. AT-III and UK were incubated at 37°C at approximately equimolar concentrations, 50-μl aliquots removed at designated time intervals, and the reaction terminated by mixing with 50 μl of SDS-containing buffer and freezing at –70°C.

Fig. 8. Radioactivity profiles of 125I-HMW urokinase incubated at 200 U/ml in normal plasma for 0, 5, 30, and 120 min. Samples were electrophoresed on (5%) polyacrylamide gels in the presence of 0.1% sodium dodecylsulfate. Gels were sliced and counted in a gamma counter. Top of gels is on the left.
These were fractions obtained by incubating HMW-UK in plasma at a concentration of 25,000 U/ml (containing 125I-HMW-UK as a tracer) for a period of 3 hr and chromatographed on Sephacryl. Profiles were similar to those illustrated in Fig. 9 (30-min incubation).

Figure 7 illustrates the time-dependent formation of high molecular weight complex between HMW-UK (first gel) and AT-III (second gel) when incubated at 37°C for 3 hr. It was noted that although the HMW-UK preparation used in these studies was contaminated with approximately 13% LMW-UK (based on quantitative gel scans), after 2 hr of incubation, 19% of the original (0 time) HMW-UK remained uncomplexed, whereas 59% of the original (0 time) contaminating LMW-UK remained uncomplexed—the LMW-UK/HMW-UK ratio increasing from 0.16 to 0.40.

Figure 8 summarizes the results obtained in the SDS-gel slicing experiments, when HMW-UK was incubated at a concentration of 200 U/ml of normal plasma. With incubation (0–120 min), the radioactivity redistributed into two major complexes with a molecular size estimated at 100,000 and greater than 300,000.

The Sephacryl gel filtration profiles of 125I-HMW-UK, incubated in normal plasma for 0, 15, and 30 min at a concentration of 200 U/ml, are illustrated in Fig. 9. Similar profiles were obtained with LMW-UK. As expected from the electrophoretic profiles (Fig. 8), with incubation, a high (fraction A) and low (fraction B) molecular weight complex formed. Fraction C represented free (uncomplexed) UK. Table I summarizes the distribution of HMW-UK–inhibitor(s) complexes in plasma identified by precipitation with antisera to the inhibitors listed. The majority of the radioactivity represented in fraction A was precipitable with antiserum to α2-macroglobulin. Depending on the type of plasma used, variable quantities of radioactivity in fraction B were precipitable with antisera to AT-III, α1-Antitrypsin, or α2-plasmin inhibitor, indicating nonselective complexing. It was noted (Figs. 8 and 9) that concomitant with the time-dependent formation of complexes, the amount of free UK (fraction C) diminished. No attempt to quantitate the ratio of free-to-bound urokinase was made.

**DISCUSSION**

With the documentation that normal plasma⁶⁻⁸ and purified inhibitors⁹⁻¹¹ destroy the biologic activity of urokinase, we compared the inhibition rates of HMW-UK and LMW-UK by plasma and by purified AT-III. In addition, we estimated the proportions of urokinase that complexed with various inhibitors in plasma.

The rates of inhibition of HMW-UK and LMW-UK in normal plasma, at concentrations ranging from therapeutic levels to 25,000 U/ml, are depicted in Figs. 2 and 3. The apparently precipitous loss of activity at concentrations of less than 100 U/ml may be an artifact of the assay system used. One explanation may be that at such low concentrations of urokinase, the dilution of the incubation mixture, prior to adding it to the clot-lysis matrix, is minimal, thereby supplying substantial quantities of inhibitors, especially α2-plasmin inhibitor (α2PI),¹²,²³ which would serve to inhibit the generated plasmin in the assay mixture. The data presented in Fig. 4 support this

<table>
<thead>
<tr>
<th>With Antiserum to</th>
<th>Percent Precipitable Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction A*</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>86–90</td>
</tr>
<tr>
<td>Antithrombin-III</td>
<td>0–6</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>α2-Plasmin inhibitor</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*These were fractions obtained by incubating HMW-UK in plasma at a concentration of 25,000 U/ml (containing 125I-HMW-UK as a tracer) for a period of 3 hr and chromatographed on Sephacryl. Profiles were similar to those illustrated in Fig. 9 (30-min incubation).
INHIBITION OF UROKINASE IN PLASMA

thesis. With the initial load of 25 U/ml, the decay of urokinase activity in normal and in plasminogen-depleted plasma is essentially the same, exhibiting the apparent precipitous loss of activity. With subsequent additions of urokinase, this apparently rapid inhibitory phase disappears in normal plasma but not in plasminogen-depleted plasma, suggesting that in normal plasma, during the incubation, the generated plasmin depletes the fast-acting α2PI thereby minimizing its effect on the plasmin generated in the assay mixture.

A second explanation may be that in normal plasma, with the initial addition of UK, plasmin is generated (an event that does not occur in plasminogen-depleted plasma), with some remaining uncomplexed with α2PI thereby influencing the lysis times obtained with the second and third addition of UK (the short lysis times interpreted as high UK activity). We consider this explanation less tenable for the following reason: At the end of the first 60 min, lysis times are practically immeasurable (greater than 3000 sec, working range 300–800 sec), indicating the presence of an immeasurable quantity of plasmin (less than 0.025 U/ml). Based on a standard plasmin assay performed in the same system, the minimum amount of plasmin required to influence our assay is about 0.32 U/ml. In spite of the technical problem described, it is evident that at all concentrations, HMW-UK is inhibited more rapidly than LMW-UK.

To confirm the role of AT-III in the inhibition of urokinase in plasma, both HMW-UK and LMW-UK were incubated in normal and in AT-III-depleted plasma, in the presence and absence of heparin. As expected (Fig. 5), heparin, at 3 U/ml (and up to 15 U/ml—not illustrated), accelerated the reaction but not dramatically. This is in contrast to the report by Clemmensen,11 who stated that heparin at 18 U/ml “greatly” accelerated the reaction between urokinase and the inhibitor. Heparin alone (up to 10 U/ml) did not inhibit UK to a significant degree.

Removal of AT-III from plasma by immunoadsorption resulted in a reduction in inhibitory capacity (Fig. 5). The observation that heparin also accelerated (slightly) the residual inhibitory potential remains unexplained. Perhaps, heparin cofactors exist in plasma other than AT-III, or residual (trace) quantities of AT-III may account for this. Our results, as well as those of Clemmensen,11 confirm the report of Tsapogas and Flute,8 who noted, in a small series of patients, that when heparin was administered concomitantly with urokinase, the fibrinolytic response was less than that seen with urokinase alone.

In the presence and in the absence of heparin, HMW-UK, at equimolar concentrations with purified AT-III, is inhibited more rapidly than LMW-UK, and in both instances, complexes, undissociable in the presence of SDS, are formed (Figs. 6 and 7). The bottom quadrant of Fig. 7 also illustrates the preferential inhibition of HMW-UK by purified AT-III. This is evidenced by the observation that the contaminating LMW-UK in the HMW-UK preparation used for the experiment complexed more slowly than HMW-UK (see Results). The heterogeneity of the complexes may be attributed to the presence of contaminating LMW-UK (~13%) and perhaps to the formation of multimers. In the course of performing these studies, we did note a certain degree of polymerization and of autolysis of HMW-UK, thereby perhaps accounting for the apparent heterogeneity of the “purified” product.

As expected from the studies performed with purified inhibitors,9–11 it is evident that, with incubation in normal plasma, HMW-UK, as well as LMW-UK (not illustrated), concomitantly with losing activity, form inactive complexes with several inhibitors (Figs. 8 and 9 and Table 1). These complexes range in molecular size from about 100,000 to greater than 300,000. The high molecular weight complex, noted as fraction A in Fig. 9, was identified by selective immunoprecipitation as a complex of urokinase and α2-macroglobulin. Approximately 90% of the radioactivity in this fraction was precipitable with antiserum to α2-macroglobulin (Table 1).

The lower molecular weight complex, noted as fraction B in Fig. 9, was similarly identified as a mixture of complexes between urokinase and AT-III or α1-antitrypsin. It is worthy of note that in AT-III-depleted plasma, as one might expect, α1-antitrypsin acquires a more significant role in the inhibition of urokinase. Similarly, in plasminogen-depleted plasma, α2-plasmin inhibitor22,23 inhibits urokinase. This is not evident in normal plasma. We conclude that α2-macroglobulin, antithrombin III, α1-antitrypsin, and perhaps other as yet unidentified serine protease inhibitors normally found in plasma, play a significant role in the inhibition of both HMW-UK and LMW-UK, and that the HMW species is inhibited slightly more rapidly than the LMW species.

Some investigators24 have questioned the relative therapeutic effectiveness of HMW-UK and LMW-UK when patients are dosed as per presently labeled activity values. While it is true that, in vitro, at normal (plasma) plasminogen concentration, when assayed against a mixed standard, one (labeled) unit of HMW-UK displays a somewhat “higher activity” than one (labeled) unit of LMW-UK by a factor of ~1.3 (our observations), this effect was not evident (nor would one expect it) in the gross clinical response of patients treated for pulmonary embolism in the Urokinase Pulmonary Embolism Trials (UPET) and
Urokinase-Streptokinase Pulmonary Embolism Trials (USPET).\textsuperscript{25-27} In both of these trials, two sources of urokinase were utilized. One product was primarily HMW-UK and the other approximately a 50/50 mixture of HMW and LMW-UK. When the two products were administered at equal dosage (activity independently assigned), no significant differences were noted in pharmacologic response and laboratory parameters. These included: bleeding, euglobulin lysis times, platelet changes, fibrinogen depletion, and plasminogen utilization. Our studies, indicating a preferential inhibition of the high molecular weight species, may help explain the apparent therapeutic equivalency of the two products, in vitro data notwithstanding.

REFERENCES

10. Clemmensen I, Christensen F: Inhibition of urokinase by complex formation with human $\alpha_1$-antitrypsin. Biochim Biophys Acta 429:591, 1976
The inhibition of high and low molecular weight urokinase in plasma

G Murano, D Aronson, L Williams and L Brown