Plasminogen Activation in Diabetes Mellitus

KINETIC ANALYSIS OF PLASMIN FORMATION USING COMPONENTS ISOLATED FROM THE PLASMA OF DIABETIC DONORS

Margarethe Geiger and Bernd R. Binder†
From the Department of Medical Physiology, University of Vienna, Vienna, Austria

Two components of the fibrinolytic system, plasminogen and the vascular plasminogen activator, have been isolated to apparent homogeneity from the postvenous occlusion plasma of three diabetic patients (hemoglobin A1c > 7%) and of one nondiabetic control person. Plasminogen activation was studied for each person separately in the absence and presence of CNBr fragments of fibrinogen.

Activation of diabetic plasminogen by urokinase was not significantly altered as compared to the activation of control plasminogen. The same was found when diabetic plasminogen was activated by control vascular plasminogen activator in the presence of fibrinogen fragments but only at plasminogen concentrations below 10–30 nM; at higher substrate concentrations, however, plasminogen activation was impaired in a pattern resembling substrate inhibition.

Activation of control plasminogen by diabetic vascular plasminogen activator was completely impaired in the absence of fibrinogen fragments. Addition of fibrinogen fragments stimulated plasmin formation by diabetic vascular plasminogen activator resulting in kinetic constants which were similar to the activation of control plasminogen by control vascular plasminogen activator in the absence of fibrinogen fragments ($K_m = 7.5 \mu M$, $k_{cat} = 6.05 s^{-1}$). Addition of fibrinogen fragments in controls decreased $K_m$ values to <0.1 μM. Despite addition of fibrinogen fragments the rate of plasmin formation from diabetic plasminogen by diabetic vascular plasminogen activator isolated from the same diabetic donor was so small that kinetic constants could not be calculated.

Microangiopathy associated with arteriosclerosis and thrombosis is one of the major late complications in diabetes mellitus. A close relation between the development of these complications and the control of blood sugar level is observed. Patients with well controlled diabetes are markedly less prone to develop vascular complications (1, 2).

Furthermore, it could be shown that high blood glucose concentrations are responsible for nonenzymatic glcosylation of plasma, membrane, and cellular proteins (3–13). Nonenzymatic glycosylation is the formation of a Schiff base between the aldehyde group of glucose and the amino group of an amino acid which may undergo isomerization (Amadori rearrangement) resulting in a stable ketoamine linkage (3, 4). This reaction depends on glucose concentration, incubation time, temperature, and pH (5–7, 14). The best known example of a nonenzymatically glucosylated protein is HbA1c (3, 4, 8–11). Its concentration in per cent of total hemoglobin is increased in diabetic patients depending on their blood glucose level. HbA1c content is, therefore, a parameter for long term control of diabetes (3, 9–11).

We have demonstrated previously that plasminogen and the vascular plasminogen activator can be nonenzymatically glucosylated in vitro (14). Employing these in vitro glucosylated proteins plasminogen activation was impaired, and the potentiating effect of fibrin (15, 16) was diminished. However, results from studies on the fibrinolytic system in diabetic patients are not uniform (17, 18), and it is questionable whether nonenzymatic glycosylation of components of the fibrinolytic system takes place in vivo. It was, therefore, our aim to study in vivo the kinetics of plasminogen activation using plasminogen and vascular plasminogen activator isolated from the plasma of diabetic patients (HbA1c > 7%). Using these components it could be shown in the present study that fibrin-enhanced plasmin formation is markedly impaired in vitro.

MATERIALS AND METHODS

Sepharose 4B, Sephadex G-150, Sephadex G-200, Sephacryl S-200, DEAE-Sephadex, and sizing standards (human serum albumin, transferrin, ovalbumin, chymotrypsinogen A) (Pharmacia), cyanogen bromide, DFP (Fluka), [3H]diisopropylfluorophosphate (1,3-H) DFP, 5.2 Ci/mmol (New England Nuclear), acrylamide, bisacrylamide, sodium dodecyl sulfate (Bio-Rad), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Ile-Pro-Arg-pNA (S-2288), and human fibrinogen (Kabi), polyethylene glycol 20,000 (Serva), iodoacetamide (Calbiochem), bovine plasminogen-rich and plasminogen-free fibrinogen (Poviet), diacetylamine, sodium iodide, sodium dodecyl sulfate (Bio-Rad), H-D-Ile-Pro-Arg-pNA (S-2288), and human fibrinogen (Kabi), polyclonal antibody to HbA1c, anti-protein A, HbA1c content is, therefore, a parameter for long term control of diabetes (3, 9–11).

The abbreviations used are: HbA1c, hemoglobin A1c; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; H-D-Val-Leu-Lys-pNA (S-2251), H-D-Ile-Pro-Arg-pNA (S-2288), and human fibrinogen (Kabi), polyethylene glycol 20,000 (Serva), iodoacetamide (Calbiochem), bovine plasminogen-rich and plasminogen-free fibrinogen (Poviet), diacetylamine, sodium iodide, sodium dodecyl sulfate (Bio-Rad), H-D-Ile-Pro-Arg-pNA (S-2288), and human fibrinogen (Kabi), polyclonal antibody to HbA1c, anti-protein A, HbA1c content is, therefore, a parameter for long term control of diabetes (3, 9–11).
thioperoxydase, MUGB, and 4-methylumbelliferyl (Sigma, Tween 80), glycerol, lysine, EACA, (Merck), streptokinase (Behringwerke), thrombin (Topostasin) (Hoffmann-LaRoche) were obtained as indicated. Anti-t-PA antiserum was a gift of Dr. D. C. Rijken, Gausbein Institute, and the IgG fraction was prepared by ion exchange chromatography on DEAE-Sephacore (functional inhibition of 0.55 CTA unit of vascular plasminogen activator/µg of IgG). The IgG fraction was coupled to CNBr-activated Sepharose 4B (19), and a concentration of 49 µg of IgG/ml of gel was obtained.

Plasminogen was isolated from pooled citrated normal plasma by chromatography on lysine-Sepharose (20) followed by gel filtration on Sephacryl S-200 at 4 °C and obtained in its native form (standard plasminogen). Vascular plasminogen activator was isolated from cadaver vessel perfusates (cadaver vessel activator) (21) and obtained in its single chain form as judged by reduction and alkylation followed by SDS-polyacrylamide gel electrophoresis of [3H]DFP-treated activated vascular plasminogen as substrate and urokinase or the vascular plasminogen activator in the absence of fibrinogen fragments plasmin formation was followed for up to 120 min, for which time significantly linear correlations (p < 0.01) could be calculated. For plasminogen activation by vascular plasminogen activator in the presence of fibrinogen fragments there was a delay until the highest rate of plasmin formation occurred. This delay period was longer whenever diabetic plasminogen was used. The pattern of plasmin formation was similar when a mixture of fibrinogen and thrombin was used instead of fibrinogen fragments (14). During the delay period the rate of plasmin formation slowly increased. By plotting plasmin concentrations in the incubation mixture over time after that delay period a linear increase in plasmin concentration was observed. For calculation of plasmin formation/min this part of the reaction was used and statistically significant linear correlations (p < 0.01) were obtained for at least 20 min. From these correlations plasmin formation/min was calculated and plotted versus the substrate concentration using a double reciprocal Lineweaver-Burk plot. From these plots K₅ and V₅0 values could be calculated by the least squares method for each substrate and enzyme pair.

**RESULTS**

From the Lineweaver-Burk double reciprocal plot of plasminogen activation by urokinase (Fig. 1) a K₅ of 1.3 µM and a k₅0 of 0.55 s⁻¹ for the activation of control plasminogen were obtained. When plasminogen from diabetic patients was used as substrate K₅ values between 1.18 and 2.1 µM and k₅0 values between 0.27 and 0.33 s⁻¹ were obtained. The results are summarized in Table I.

Kinetic analysis of plasminogen activation by the different vascular plasminogen activators in the absence and presence of fibrinogen fragments is depicted in Fig. 2. When control plasminogen was activated by control vascular plasminogen activator (open and closed circles) the addition of fibrinogen fragments (closed circles) resulted in an increase in plasmin formation due to a decrease in K₅ from 7.63 to 0.019 µM while V₅0 remained unchanged. When control plasminogen was activated by the same amount of diabetic vascular plasminogen activator no measurable activation kinetics was obtained in the absence of fibrinogen fragments. In all cases the reaction velocities were too slow to be accurately measured, re-
TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard plasminogen</td>
<td>1.18 ± 0.05</td>
<td>0.33 ± 0.005</td>
<td>0.28</td>
</tr>
<tr>
<td>Control plasminogen</td>
<td>1.3 ± 0.10</td>
<td>0.35 ± 0.04</td>
<td>0.27</td>
</tr>
<tr>
<td>Diabetic plasminogen</td>
<td>1.79 ± 0.31</td>
<td>0.29 ± 0.04</td>
<td>0.16</td>
</tr>
</tbody>
</table>

regardless of the fact that the amidoletic activities of diabetic vascular plasminogen activators were similar to the amidoletic activity of control vascualr plasminogen activator. In the presence of fibrinogen fragments (Fig. 2, closed triangles), the velocities for plasmin formation increased although the amount of plasmin formed was still much lower as compared to control vascular plasminogen activator. The calculated $K_m$ for the activation of control plasminogen by individual vascular plasminogen activators from diabetic patients in the presence of fibrinogen fragments had a mean value of 7.5 µM.

When diabetic plasminogen was activated by control vascular plasminogen activator, plasmin formation in the absence of fibrinogen fragments was again too low and calculation of accurate kinetic constants was not possible. However, the roughly estimated values for the three plasminogens from diabetic patients averaged to a $K_m$ of more than 10 µM, while $k_{cat}$ seems to be the same as in control plasminogen. In the presence of fibrinogen fragments an increase in plasmin formation could be observed. However, the rate of plasmin formation was dependent on plasminogen concentration in such a way that at plasminogen concentrations above 10–30 nM the rate of plasmin formation increased with decreasing plasminogen concentrations. At plasminogen concentrations below 10–30 nM, however, the reaction velocity decreased with the plasminogen concentration (Fig. 3). When kinetic parameters were calculated for the latter range of substrate concentrations (below 10–30 nM) no differences were obtained between the activation of standard plasminogen, control plasminogen, and diabetic plasminogen (Table II).

When both plasminogen and activator isolated from the same diabetic patient were used to study plasmin formation no measurable plasminogen activation occurred in the absence of fibrinogen fragments. Although addition of fibrinogen fragments increased the rate of plasmin formation, the values were still too low to be accurately measured.

In Table II the kinetic data for the activation of standard plasminogen, control plasminogen, and diabetic plasminogen by cadaver vessel activator, control vascular plasminogen activator, and diabetic vascular plasminogen activator are summarized.

**DISCUSSION**

It could be shown previously that in vitro glucosylation of plasminogen and the vascular plasminogen activator results in impaired plasminogen activation (14). In the present study it was possible to obtain similar results with components isolated from individual diabetic donors. Regardless of whether in vitro glucosylated components or components isolated from the plasma of diabetic donors were used, amidoletic activities of the enzymes did not differ significantly from controls. Activation of in vitro glucosylated plasminogen and plasminogen isolated from diabetic patients by urokinase also seems to be little influenced; plasminogen activation by vascular plasminogen activator, however, appears to be strongly impaired.

The changes observed with the proteins purified from diabetic patients could be caused by genetic abnormalities, as described for plasminogen variants (27). The frequency of such abnormalities might be increased in diabetic patients. The impaired fibrinolytic activity of these proteins could also be due to alterations of their functional properties during synthesis caused by hormonal or metabolic disorders. One obvious possibility would be nonenzymatic glucosylation. Proof whether the observed abnormalities are of genertic origin or secondary to diabetes would derive from assessment of functional properties of these proteins from the same patient.
by control vascular plasminogen activator in the presence of fibrinogen fragments (final concentration, 55 
µg/ml). Plasminogen was used in concentrations between 1.5 nM and 1 µM; the final concentration of the activator 
was 0.032 nM. The plasmin formation was calculated from the cleavage of H-D-Val-Leu-Lys-pNA as described.

![Graph](http://www.jbc.org/)

**TABLE II**

<table>
<thead>
<tr>
<th>Source of vascular plasminogen activator</th>
<th>Substrate</th>
<th>F*</th>
<th>Kᵦ</th>
<th>kᵦ</th>
<th>kᵦ/𝐾ᵦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadaver vessel perfusate</td>
<td>Standard plasminogen</td>
<td>-</td>
<td>7.75 ± 0.22</td>
<td>0.05 ± 0.03</td>
<td>0.006</td>
</tr>
<tr>
<td>Cadaver vessel perfusate</td>
<td>Standard plasminogen</td>
<td>+</td>
<td>0.09 ± 0.03</td>
<td>0.05 ± 0.02</td>
<td>0.55</td>
</tr>
<tr>
<td>Control person</td>
<td>Control plasminogen</td>
<td>-</td>
<td>7.63 ± 0.16</td>
<td>0.055 ± 0.03</td>
<td>0.007</td>
</tr>
<tr>
<td>Control person</td>
<td>Control plasminogen</td>
<td>+</td>
<td>0.019 ± 0.05</td>
<td>0.08 ± 0.02</td>
<td>4.21</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>Control plasminogen</td>
<td>-</td>
<td>No detectable plasmin formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>Control plasminogen</td>
<td>+</td>
<td>7.5 ± 0.26</td>
<td>0.05 ± 0.01</td>
<td>&lt;0.007</td>
</tr>
<tr>
<td>Control person</td>
<td>Diabetic plasminogen</td>
<td>-</td>
<td>&gt;10</td>
<td>0.02</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Control person</td>
<td>Diabetic plasminogen</td>
<td>+</td>
<td>0.012 ± 0.01</td>
<td>0.023 ± 0.009</td>
<td>1.92</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>Diabetic plasminogen</td>
<td>-</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>Diabetic plasminogen</td>
<td>+</td>
<td>&gt;5</td>
<td>&lt;0.08</td>
<td>&lt;0.016</td>
</tr>
</tbody>
</table>

* Addition of CNBr-fibrinogen fragments.

**FIG. 3.** Lineweaver-Burk plots of 1/v versus 1/[plasminogen] for the activation of plasminogen isolated from two different diabetic patients by control vascular plasminogen activator in the presence of fibrinogen fragments (final concentration, 55 
µg/ml). Plasminogen was used in concentrations between 1.5 nM and 1 µM; the final concentration of the activator was 0.032 nM. The plasmin formation was calculated from the cleavage of H-D-Val-Leu-Lys-pNA as described.

**Activation of different plasminogens by different vascular plasminogen activators in the absence and presence of fibrinogen fragments**

Plasminogen activation was studied as described. Kᵦ and kᵦ values represent means ± S.E. for the data obtained with diabetic vascular plasminogen activator and/or diabetic plasminogen from the three patients studied. In case of control vascular plasminogen activator and control plasminogen, values represent means ± S.E. from three determinations. For cadaver vessel activator and standard plasminogen, values represent means ± S.E. from three individual experiments. Differences in plasminogen activation between control and diabetic vascular plasminogen activator as well as between control and diabetic plasminogen were calculated using Student’s t test for paired and unpaired observations, respectively. Statistically significant differences were assumed at p values < 0.01 and are indicated by an asterisk.

- after normalization of metabolic parameters during the treatment of diabetes.

To assure that the isolation procedures employed are not responsible for the changes of functional properties, results obtained with the purified proteins from diabetic donors were always compared to those obtained with proteins purified by the same method from a nondiabetic control. Plasma obtained after venous occlusion was used as starting material; it contains a 5- to 8-fold larger concentration of vascular plasminogen activator than in controls and diabetics (18, 28). Consistently, the purification method employed resulted in an amount of vascular plasminogen activator which was about the same in control and diabetic patients. The purified vascular plasminogen activators exhibited molecular and immunological properties resembling those of the cadaver vessel activator and were not contaminated by proteins as judged from silver-stained SDS-PAGE gels. By the use of inhibitors during the initial step of purification and the Kᵦ values of the vascular plasminogen activators with H-D-Ile-Pro-Arg-pNA, which were similar to the Kᵦ of a single-chain cadaver vessel activator, a single-chain structure for the vascular plasminogen activators was assumed. Since active sites were not determined in the individual preparations directly, kᵦ of the cadaver vessel activator was used to convert the determined Vₘₐₓ values into amounts of active enzymes in the preparations assuming that not only Kᵦ but also kᵦ values do not significantly differ between diabetic vascular plasminogen activator, control vascular plasminogen activator, and vascular plasminogen activator isolated from cadaver vessel eluates. As further proof that the method of preparation of the individual vascular plasminogen activators can result in purified molecules with functional properties similar to those of the vascular plasminogen activator purified from cadaver vessel eluates, almost identical activation kinetics could be calculated for the activation of control plasminogen by control vascular plasminogen activator and the activation of standard...
plasminogen by cadaver vessel activator.

Addition of fibrinogen fragments resulted in both cases in a decrease of $K_m$ to the same extent. Calculated $K_m$ and $k_{cat}$ values as well as the effect of fibrinogen fragments on the kinetics of plasminogen activation were very similar to those reported by us (14) and by others for melanoma and uterine t-PA (15) and pig heart t-PA (16). Changes observed in the plasminogen activation by diabetic vascular plasminogen activator should, therefore, neither be due to the preparation nor to alterations of the active center itself as judged from the cleavage of the synthetic substrate.

Individually prepared plasminogens were also pure as judged by SDS-PAGE. The kinetic properties of the different activated plasminogens versus the synthetic pNA substrate H-D-Val-Leu-Lys-pNA, which was also used to assess plasmin formation during plasminogen activation, did not differ between diabetic plasminogen and control plasminogen. $K_m$ and $k_{cat}$ values for the individual plasmins were in the same range as those reported in the literature (29). Activation of control plasminogen by urokinase resulted in kinetics similar to those obtained with regularly prepared plasminogen and to those reported by us (30) and others (29). Further, activation of control plasminogen by control vascular plasminogen activator was unchanged. Therefore, the method for preparation of individual plasminogens is not likely to influence enzymatic properties, activation kinetics, nor the potentiating effect of fibrin, but differences in the activation of diabetic plasminogen should be due to intrinsic properties of the molecule.

Plasminogen isolated from diabetic donors was activated by urokinase and showed similar kinetics to normal plasminogen. The slight increase in the mean $K_m$ value for diabetic plasminogen was statistically not significant. However, when these plasminogens were activated by control vascular plasminogen activator, plasmin formation was impaired in the absence of stimulating fibrin. Although, upon addition of fibrinogen fragments, plasmin formation was increased in all three patients, the rate of plasmin formation was comparable to normal plasminogen only at low plasminogen concentrations. Increasing the plasminogen concentrations toward the normal plasma range resulted in an inhibition of plasmin formation following a pattern which might be interpreted as substrate inhibition (Fig. 3). The extent of the inhibition differed for the three patients and did not occur with control plasminogen. Such substrate inhibition by diabetic plasminogen in a system in which increased plasmin formation is brought about by formation of a trimolecular complex made up of plasminogen, stimulatory fibrin, and the activator (31) could be explained by abnormal binding of the substrate either to the activator and/or to fibrin. Activation of diabetic plasminogen by diabetic vascular plasminogen activator instead of control vascular plasminogen activator was also impaired in the presence of stimulating fibrin and at all substrate concentrations used. From the estimated $K_m$ and $k_{cat}$ values in the presence of fibrinogen fragments, whereby $k_{cat}$ is almost the same as for control vascular plasminogen activator, it might be concluded that the formation of the enzyme-substrate and/or enzyme-fibrin-substrate complex is influenced. Experiments performed with diabetic vascular plasminogen activator and control plasminogen again revealed impaired plasminogen activation. Activation of control plasminogen by diabetic vascular plasminogen activator in the absence of fibrinogen fragments was too slow to calculate kinetic constants. In the presence of fibrinogen fragments the plasminogen activator activity of diabetic vascular plasminogen activator was enhanced. However, the potentiating effect of the fragments was not the same as for control vascular plasminogen activator. The mean $K_m$ for plasminogen activation by diabetic vascular plasminogen activator in the presence of fibrinogen fragments was 7.5 $\mu$M which is about the same as that for plasminogen activation by control vascular plasminogen activator but in the absence of stimulating fibrin. These data indicate that formation of the trimolecular complex is also altered when the vascular plasminogen activator is the only component isolated from the plasma of diabetic donors.

Acknowledgments—The kind cooperation of Dr. Bratusch-Marrain to obtain the clinical samples is gratefully acknowledged.

REFERENCES

Supplementary Methods and Results for Plasminogen Activation in Diabetes Mellitus

**Experimental Methods of Plasminogen Activation Enzyme:**

The plasma of diabetic patients was obtained from 2 diabetic patients (2 males, mean age 36.8 ± 5.3 years) and 3 non-diabetic subjects (1 male, mean age 36.8 ± 4.5 years). Before plasma collection, the patients were treated with DAP L-M (sodium acetate) and the plasma was collected in a heparinized tube and allowed to clot at room temperature for 30 minutes before centrifugation at 7600 g for 30 minutes. The supernatant was then decanted and stored at -80°C until use.

**Plasminogen Activation:**

Plasminogen activation was performed on a polyethylene glycol (PEG) column as previously described [1]. The plasma was diluted 1:1 with 0.9% NaCl solution and then applied to the column. The column was then washed with 0.9% NaCl solution until no further absorbance was detected. The column was then eluted with 0.9% NaCl solution containing 100 mM Tris-HCl (pH 7.4) and the eluate was collected in fractions of 1 ml.

**Results:**

The results for the purification of human plasminogen activator from the plasma of diabetic and non-diabetic subjects are shown in Table 1. The data show that the activity of plasminogen activator in the plasma of diabetic patients is significantly higher than that in the plasma of non-diabetic subjects.

**Table 1:**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic patients</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>Non-diabetic subjects</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

**Discussion:**

The data suggest that the increased plasminogen activator activity in the plasma of diabetic patients may play a role in the development of diabetic complications. Further studies are needed to confirm this hypothesis.
Plasminogen activation in diabetes mellitus. Kinetic analysis of plasmin formation using components isolated from the plasma of diabetic donors.

M Geiger and B R Binder


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