Cerebral Plasminogen Activator Activity in Spontaneously Hypertensive Stroke-Prone Rats

Osamu Matsuo, MD; Kiyotaka Okada, PhD; Hideharu Fukao, PhD; Aritomo Suzuki, MD; and Shigeru Ueshima, MD

Background and Purpose: In the spontaneously hypertensive stroke-prone rat, it is unclear whether plasminogen activator plays a role in the development of stroke. The present study was undertaken to investigate brain levels of plasminogen activator activity in spontaneously hypertensive stroke-prone rats and Wistar-Kyoto rats.

Methods: Plasminogen activator was purified from the brains of rats of both strains. The purification involved used ammonium sulfate precipitation, gel filtration, a zinc chelate–Sepharose column, and a concanavalin A–Sepharose column. Fraction I (0.15 M KCl-soluble fraction) and fraction II (2 M KCl plus 6 M urea-soluble fraction) were purified from both strains.

Results: Total plasminogen activator activity in the original homogenates for fractions I and II derived from spontaneously hypertensive stroke-prone rats was increased to twice the level found in Wistar-Kyoto rats. The final product purified from fractions I and II in both strains of rats revealed single bands of plasminogen activator activity on enzymatic analysis with a molecular weight of 72,000. The purified product had stronger S-2288 amidolytic activity than S-2444 amidolytic activity, and it also displayed fibrin-binding ability.

Conclusions: The study demonstrated that there is an increased content of plasminogen activator in the brains of spontaneously hypertensive stroke-prone rats that might be related to the development of stroke.

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KEY WORDS • hypertension • plasminogen activators • rats

Plasminogen activator is a serine protease that converts plasminogen to plasmin, and the plasmin so produced can then catalyze fibrin or fibrinogen. Decreased levels of plasminogen activator activity are observed in some hereditary conditions and can cause thrombosis.1-2 Conversely, the increase in plasminogen activator activity that often occurs after the administration of large doses of urokinase (urinary plasminogen activator), tissue-type plasminogen activator (t-PA), anisoylated plasminogen-streptokinase activator complex, or streptokinase can cause a hemorrhagic tendency.3-5 Thus, the plasminogen activator level must be regulated within a certain limited range under physiological conditions to maintain normal blood coagulability. The spontaneously hypertensive stroke-prone rat (SHR-SP) develops hypertension and dies of cerebral stroke.6-7 Whether such strokes are induced by an increase of plasminogen activator activity still remains unclear, and the pathophysiological role of the fibrinolytic system in cerebral stroke requires further elucidation. The present study was undertaken to investigate plasminogen activator activity in the brains of SHR-SP in comparison with Wistar-Kyoto rats (WKY), the strain from which SHR-SP were derived.

Materials and Methods

We used 6-month-old male SHR-SP and WKY (both supplied by the Animal House at the Kinki University School of Medicine.) The brains were obtained after decapitation, with the skull, dura, and vessels removed. The cerebellum was also removed, and the cerebrum was stored at −80°C until use. Six rats of each strain were used for each experiment, and the purification procedures were performed in three separate experiments as follows. After thawing, the brains were washed with phosphate-buffered saline, minced in a 20-fold excess (by weight) of 0.15 M KCl, and homogenized in a sonicator (Ultrasonic, W-220F). After centrifugation at 6,000 rpm for 30 minutes, the supernatant (fraction I) was used for the subsequent purification steps, and the pellet was resolubilized in 2 M KCl and 6 M urea (the same volume as used for the initial homogenate) to give fraction II.

Both fractions I and II were treated with ammonium sulfate (30%, 50%, and 70%), which with the strongest plasminogen activity was subjected to further purification. After dialysis for 10 hours with 0.1 M Tris-HCl (pH 7.5) containing 1 M NaCl and 0.01% Tween 80, the fraction was subjected to Sephadex G-25 gel filtration. The fraction with the protein peak was then applied to a Zn chelate–Sepharose column (Pharmacia) which had been previously chelated with ZnCl₂ (5 mg/ml), and was equilibrated with 0.1 M Tris-HCl.
TABLE 1. Purification of Plasminogen Activator from Brains of WKY and SHR-SP

<table>
<thead>
<tr>
<th></th>
<th>Original homogenate</th>
<th>Precipitated with (NH₄)₂SO₄</th>
<th>Adsorbed to Zn chelate-Sepharose</th>
<th>Adsorbed to concanavalin A-Sepharose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WKY groups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I</td>
<td>51.9 (100.0%)</td>
<td>57.0 (109.8%)</td>
<td>76.0 (146.4%)</td>
<td>55.0 (105.9%)</td>
</tr>
<tr>
<td></td>
<td>0.097 (1)</td>
<td>0.155 (1.60)</td>
<td>13.2 (135.7)</td>
<td>108.7 (1,121)</td>
</tr>
<tr>
<td>Fraction II</td>
<td>97.5 (100.0%)</td>
<td>41.2 (42.2%)</td>
<td>54.1 (55.4%)</td>
<td>51.9 (53.2%)</td>
</tr>
<tr>
<td></td>
<td>0.044 (1)</td>
<td>0.128 (2.91)</td>
<td>8.49 (193.0)</td>
<td>98.8 (2,245)</td>
</tr>
<tr>
<td><strong>SHR-SP groups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I</td>
<td>121.8 (100.0%)</td>
<td>84.9 (67.9%)</td>
<td>78.2 (62.6%)</td>
<td>55.0 (44.0%)</td>
</tr>
<tr>
<td></td>
<td>0.189 (1)</td>
<td>0.234 (1.24)</td>
<td>12.7 (67.0)</td>
<td>113.9 (603)</td>
</tr>
<tr>
<td>Fraction II</td>
<td>201.7 (100.0%)</td>
<td>50.6 (10.2%)</td>
<td>95.5 (47.3%)</td>
<td>98.9 (49.0%)</td>
</tr>
<tr>
<td></td>
<td>0.071 (1)</td>
<td>0.106 (1.49)</td>
<td>17.4 (245.1)</td>
<td>216.8 (3,054)</td>
</tr>
</tbody>
</table>

Fraction I, soluble in 0.15 M KCl; Fraction II, soluble in 2 M KCl plus 6 M urea. Total activity, yield, specific activity, and purification factor of fractions I and II at each step in purification are shown. Yield is shown in parentheses in upper lines and was calculated by setting total activity in original homogenate as 100%. Purification factor shown in parentheses in lower lines was calculated by taking specific activity in original homogenate as 1. Values for total activity (first number in upper lines) are in international units; values for specific activity (first number in lower lines) are IU/A₂₈₀. WKY, Wistar-Kyoto rat; SHR-SP, spontaneously hypertensive stroke-prone rat.

(pH 7.5) containing 1 M NaCl and 0.01% Tween 80. After washing the column with the above-mentioned buffer, the bound protein was eluted with 0.1 M Tris-acetate (pH 5.5) containing 1 M NaCl and 0.01% Tween 80. The eluted fraction (1 ml/tube) was immediately neutralized with 50 μl of 1.5 M Tris-HCl (pH 7.5). The fraction with plasminogen activator activity was further applied to concanavalin A-Sepharose that had been previously equilibrated with 0.1 M Tris-HCl (pH 7.5) containing 1 M NaCl and 0.01% Tween 80. After washing the column with the above-mentioned buffer, the bound protein was eluted with the same buffer plus 0.4 M methyl β-D-mannopyranoside and 0.6 M potassium thiocyanate. The fraction with plasminogen activator activity was then employed for the characterization of its chromogenic activity and fibrin affinity. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed essentially as described by Weber and Osborn. Plasminogen activator activity was measured by the fibrin film method as well as by electrophoretic enzymography. Electrophoretic enzymography was performed with sodium dodecyl sulfate–polyacrylamide gel containing plasminogen-rich fibrin. After electrophoresis, the gel was placed in 2.5% Triton X-100 and washed three times. It was then incubated in 0.1 M glycine-NaOH buffer (pH 8.3) at 37°C and stained with Coumassie brilliant blue.

Fibrin affinity was investigated using fibrin-Sepharose that had been previously equilibrated with 0.1 M Tris-HCl (pH 7.5) containing 0.01% Tween 80. After washing the column with the above-mentioned buffer, the bound portion was eluted first with the same buffer containing 0.2 M arginine and again with the buffer containing 0.2 M arginine, 2 M potassium thiocyanate, and 6 M urea.

The amidolytic activity of the purified sample was measured using Pyroglu-Gly-Arg-pNA (S-2444) and H-D-Ile-Pro-Arg-pNA (S-2288) (KABI Diagnostica). Samples (50 μl) were serially diluted with 0.1 M Tris-HCl (pH 7.5) containing 0.01% Tween 80 and were mixed with 100 μl of 0.1 M Tris-HCl (pH 8.4) plus 0.1 M NaCl in a 96-well microplate. Then 50 μl 1 mM chromogenic substrate was added, and the increase in optical density at 405 nm was measured with a dual-wavelength microplate photometer (Corona Electric, MTP-22, Tokyo). Plasminogen activator was purified from the tissue culture medium of a melanoma cell line (Bowes) or was obtained from fresh urine or urokinase, respectively for use as the control. The polyclonal antibody against t-PA or urokinase was prepared in rabbits as described elsewhere.

The plasminogen activator inhibitor activity was measured by reacting two-chain urokinase with brain homogenates from both strains of rats, which were produced by the procedure used in the purification of plasminogen activator. The activity inhibiting 1 unit of two-chain urokinase was defined as 1 unit of plasminogen activator inhibitor.

**Results**

Plasminogen activator was purified from fractions I and II in three separate experiments, and typical results are shown in Table 1. The total plasminogen activator activity in fraction I (0.15 M KCl-soluble fraction) and fraction II (2 M KCl plus 6 M urea-soluble fraction) was 51.9±6.11 IU and 201.7±16.55 IU for WKY, and 121.8±14.26 IU and 216.8±3054 IU for SHR-SP, respectively (mean±SD). There was a statistically significant difference between WKY and SHR-SP (p<0.01 for fraction I and p<0.001 for fraction II). The specific plasminogen activator activities (IU/A₂₈₀) of fractions I and II were 0.097±0.0114 and 0.044±0.0053 for WKY, and 0.189±0.0221 and 0.071±0.0058 for SHR-SP, respectively (both p<0.001).

After treatment with 30% ammonium sulfate, fraction I contained a higher plasminogen activator activity in the precipitate in both WKY and SHR-SP, whereas fraction II contained a higher plasminogen activator activity in the precipitate at 50% ammonium sulfate. Subsequently, 30% ammonium sulfate was used to precipitate fraction I and 50% ammonium sulfate for fraction II.
When the fraction containing plasminogen activator was applied to Zn chelate–Sepharose, the unbound protein passed through and contained no plasminogen activator activity (Figure 1). After changing to the elution buffer, the bound protein was eluted first, and then the plasminogen activator was eluted. The elution profiles from Zn chelate–Sepharose were similar for both WKY and SHR-SP. The fraction with plasminogen activator activity was next applied to concanavalin A–Sepharose, and the activity was eluted after the peak of bound protein. This fraction was used for further biological characterization. The pooled fraction showed one major band (>85%) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and several minor bands. It did not contain any inhibitor of plasminogen activator activity (data not shown).

The total activity, recovery rate, specific activity (IU/A280 nm), and purification factor for each step of purification are listed in Table 1. The final purification factors were about 1,100 for fraction I and 2,200 for fraction II, while the values for SHR-SP were 600 and 3,100, respectively.

The pooled fraction of concanavalin A–Sepharose bound plasminogen activator (referred to below as the purified product) was analyzed by electrophoretic enzymography. As shown in Figure 2, the active component of plasminogen activator revealed a single band with a molecular weight of 72,000, which was similar to that of t-PA. There were no differences in molecular weight among all the fractions I and II from WKY and SHR-SP.

The amidolytic activity, which was measured with two chromogenic substrates (S-2444 and S-2288), is shown.
in Figure 3. The purified product from SHR-SP fraction I exhibited the strongest S-2288 amidolytic activity, while WKY fraction II, SHR-SP fraction II, and WKY fraction I also showed S-2288 amidolytic activity in descending order (closed circles in Figure 3). These samples demonstrated little S-2444 amidolytic activity (open circles in Figure 3), indicating that the purified activator was a form of t-PA.

When the fibrin affinity of the purified product was investigated with a fibrin-Sepharose column, the t-PA bound to the fibrin-Sepharose (Figure 4A), whereas urokinase did not (Figure 4B). When the purified product of SHR-SP fraction II was applied to a fibrin-Sepharose column, approximately one third of the plasminogen activator appeared in the unbound fraction, and approximately 40% of the plasminogen activator was obtained when the bound portion was eluted with 0.2 M arginine. The remaining plasminogen activator was eluted with 2 M potassium thiocyanate and 6 M urea (Figure 4C). Thus, approximately two thirds of the plasminogen activator was bound to the fibrin-Sepharose during the second chromatography procedure. Thus, the purified product contained both fibrin-unbound and fibrin-bound plasminogen activator. Similar results were observed for the other purified products obtained from SHR-SP and WKY (Table 2). All fractions, including the fibrin-unbound portion, the 0.2 M arginine-eluted portion, and the 2 M potassium thiocyanate plus 6 M urea-eluted portion showed a single plasminogen activator band with a molecular weight of 72,000 by electrophoretic enzymography.

Discussion

The SHR-SP develops hypertension and dies of cerebral stroke. However, the role of plasminogen activator or the fibrinolytic system in this process has not previously been investigated. Our present results indicate that the SHR-SP brains contain almost double the amount of plasminogen activator as the WKY brains. Therefore, an increase in plasminogen activator activity in the SHR-SP brain might augment the risk of hemorrhagic episodes. Once a hemorrhage occurs, the high blood pressure in SHR-SP (232 ±8 mm Hg for SHR-SP versus 133±3 mm Hg for WKY at 6 months of age) as well as the consumption of hemostatic fibrin, which may be induced by higher plasminogen activator levels, would both tend to exacerbate the hemorrhagic episode. Hypertensive vascular changes and impaired fibrin formation may further contribute to the worsening of hemorrhage, although adequate data to determine this have not yet been accumulated. When high doses of plasminogen activator are administered to patients, the incidence of intracranial hemorrhage increases. Thus, if a twofold increase in brain plasminogen activator levels in SHR-SP acts synergistically with their high blood pressure, the local hemostatic system could be overcome and progression to a fatal hemorrhagic episode could occur.
The plasminogen activator content of in both fractions I and II was higher in SHR-SP than in WKY. Because fraction I is soluble under physiological conditions, 17 the release of its plasminogen activator may be rapid. Fraction II is most likely to represent a bound form of plasminogen activator, but when the activity in this fraction was assayed by electrophotoretic enzymography, its molecular weight was found to be similar to that of fraction I. The binding to other proteins may thus be weak and allow separation during electrophoresis in the presence of sodium dodecyl sulfate, as has been described previously. 18, 19

Electron microscopy 20 has revealed that subendothelial fibrin deposits cause narrowing of the arterial lumen in SHR-SP before the symptoms of stroke arise. When stroke finally occurs, massive fibrin deposition in the subendothelial space as well as in the media of occluded arteries can be observed, while the arterial lumen is filled with thrombi composed of platelets, red blood cells, and fibrin. On the other hand, there is no fibrin deposition at the sites of arterial rupture. These observations indicate that the fibrinolytic system is not activated at the sites of thrombosis, but that initially, as pathological changes develop and a hemorrhagic event is induced, 21 fibrin deposits may be lysed by activation of the fibrinolytic system. When the plasminogen activator content was compared in 4-, 12-, 24-, and 48-week-old rats, no difference between SHR-SP and WKY was observed at 4 and 12 weeks of age. However, the plasminogen activator level in SHR-SP was double that of WKY at 24 and 48 weeks of age (data not shown). The increase in plasminogen activator may thus be secondary to the increase in blood pressure, but further investigations are needed to clarify the possible role of genetic factors.

When the molecular weights of plasminogen activator were compared between SHR-SP and WKY, no difference was observed (Figure 2), suggesting that a qualitative difference did not exist. Fibrin affinity was also similar in all the fractions obtained from SHR-SP and WKY (Table 2). The physiological significance of the presence of fibrin-unbound plasminogen activator with this molecular weight is not yet clear. Because the presence of plasminogen activator inhibitor in a sample can affect the plasminogen activator activity, the level of plasminogen activator inhibitor in the brain tissue was measured previously. 22 Plasminogen activator inhibitor levels in WKY were almost identical to those in SHR-SP (11.0 IU versus 116.9 IU). This supports our finding that plasminogen activator activity in the SHR-SP brain was significantly higher than that in the WKY brain. The present results indicate that plasminogen activator in SHR-SP was quantitatively different from that in WKY, but not qualitatively different. In SHR-SP, plasminogen activator activity appears to be dominant in the brain, and a hemorrhagic tendency may be easily induced. However, because the fibrinolytic system is also affected by various coagulation factors and by platelet function, further studies are required to clarify the dynamic changes occurring in the progression from a thrombotic state to a hemorrhagic state. In particular, the enzymatic activity of plasminogen activator and plasmin has to overcome the inhibitory activity of plasminogen activator inhibitor and α2-antiplasmin to lyse fibrin, but the details of this process are also unknown. Thus, the demonstration of a higher content of plasminogen activator does not explain all the events occurring in the SHR-SP brain, but it suggests that plasminogen activator plays an important role in the progression of stroke in this strain of rats.

References

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