Involvement of Thrombolysis in Recombinant Tissue Plasminogen Activator–Induced Cerebral Hemorrhages and Effect on Infarct Volume and Postischemic Endothelial Function

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Background and Purpose—In a model of mechanical focal ischemia, we investigated the involvement of thrombolysis products (TLP) in recombinant tissue plasminogen activator (rtPA)–induced intracerebral complications and the effects on infarct volume and postischemic endothelial function.

Methods—Hemorrhage incidence and severity were evaluated by histomorphometric analysis in male spontaneously hypertensive rats (SHR) subjected to 60-minute intraluminal middle cerebral artery (MCA) occlusion and receiving intravenously 5 hours later either saline, rtPA (3, 10, or 30 mg/kg), or rtPA (10 mg/kg) associated with TLP (rtPA+TLP). In addition, MCA reactivity was assessed in rtPA- or rtPA+TLP-treated SHR versus control Wistar-Kyoto rats or SHR.

Results—No hemorrhage was observed visually in SHR receiving saline. In contrast, rtPA administration induced hemorrhagic complications in infarcted areas in a dose-independent manner. Administration of rtPA+TLP solution, containing a high concentration of plasmin, did not affect hemorrhage incidence but significantly increased hemorrhage severity (8.8±2.3 petechiae versus 3.0±1.0 petechiae in rtPA group; P<0.001). This increased severity was associated with a significant increase of both infarct volume (182±10 versus 144±15 mm³ in rtPA group; P<0.01) and postischemic impairment of MCA endothelium-dependent relaxation (9±0.5% versus 13±1% in rtPA group; P<0.05).

Conclusions—Treatment with rtPA led to intracerebral hemorrhages, in contrast to saline-treated animals, and the presence of TLP increased the severity of these hemorrhages, in parallel with increased infarct volume and worsened endothelial function. (Stroke. 2003;34:2981-2985.)

Key Words: hemorrhage ■ ischemia, focal ■ plasmin ■ thrombolysis ■ tissue plasminogen activator

Thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) has demonstrated improvement in clinical outcome in acute ischemic stroke. However, the threat of intracerebral hemorrhage (ICH) is an important barrier to widespread administration of thrombolytic agents. While many factors, such as elevated blood pressure, diabetes, age, and rtPA administration time, contribute to the occurrence of ICH, the underlying mechanisms remain elusive.

ICH are preferentially located in the infarct area, suggesting a role of ischemia per se. In response to ischemic and inflammatory stimuli, vascular modifications may also be involved in ICH pathophysiology. In particular, endothelium-dependent relaxation, a marker of ischemia-induced impairment of cerebral vasculature, is worsened in vitro by the application of rtPA.

It remains unknown whether rtPA directly induces ICH or if the interaction between rtPA and thrombus, via the resulting thrombolysis products (TLP), is involved. Existence of rtPA-induced ICH in thromboembolic models has been well established, whereas it remains more uncertain in mechanical models of ischemia. The difference between the 2 models suggests that thrombus may contribute to the occurrence of ICH. To test this hypothesis, we compared the effects of rtPA or rtPA-induced TLP in a model of mechanical middle cerebral artery (MCA) occlusion on ICH incidence and severity. We also studied infarct volume and MCA endothelial function to investigate the mechanism of ICH occurrence during rtPA-induced thrombolysis.

Materials and Methods
Male spontaneously hypertensive rats (SHR) (Elevage Janvier, France) or Wistar-Kyoto rats (WKY) (Iffa credo, France) weighing 270 to 320 g were used.
Cerebral Ischemia

Under anesthesia (chioral hydrate 300 mg/kg), focal cerebral ischemia was induced by 60-minute occlusion of the MCA by an intraluminal filament inserted in the external carotid artery to the internal carotid artery, according to a modified protocol from previously described methods. Physiological parameters (temperature, mean arterial pressure, arterial blood pH, PaO₂, and PaCO₂) were controlled throughout the experiments. Subsequently, the right jugular vein was isolated and cannulated with a polyethylene catheter (24-gauge) for intravenous rtPA infusion. Thereafter, animals were allowed to recover from anesthesia and to eat and drink freely. Sham rats underwent the same interventions, without advancement of intraluminal filament in the internal carotid artery.

Preparation of rtPA+TLP Solution

Shortly after the onset of ischemia, 0.2 mL of autologous blood was sampled by jugular vein and left in the open air for 5 hours, allowing thrombus formation. The clot was then fragmented, and rtPA 10 mg/kg (6 mL/kg) was applied on the clot pieces for 30 minutes. The resulting solution was collected. This solution mainly contained plasmin, detected by its amidolytic activity on the synthetic chymogenic substrate CBS 10.85 (pNA release at 405 nm). D-dimers, detected by enzyme-linked immunosorbent assay, were not found.

Assessment of ICH and Infarction

Rats were killed 24 hours after reperfusion. Brains were removed rapidly, frozen, and coronally sectioned into 100-μm-thick slices at 12 levels according to a stereotactic section map. ICH were identified visually on cutting sections and recorded in a blind manner as present or not. There were 2 types of hemorrhagic transformation: (1) hemorrhagic infarction, identified as petechiae, and (2) parenchymal hemorrhage, identified as hematoma. Hematoma and petechiae were counted per volume of brain, and the total number of petechiae in infarcted area estimated ICH severity. Other adjacent sections were stained with cresyl fast violet to blindly quantify petechiae in infarcted area estimated ICH severity. Other adjacent sections were stained with cresyl fast violet to blindly quantify infarct and hemispheric volumes (mm³). The unstained sections were defined as infarcted. Total, cortical, and subcortical infarct volumes and hemispheric volumes (mm³) were obtained by the use of numerical integration of the respective calculated areas for all sections per animal. A corrected volume for edema was calculated with the use of the following equation: Corrected Infarct Volume=Infarct Volume–(Right Hemisphere Volume–Left Hemisphere Volume).

Vasoreactivity Analysis

Endothelium-dependent and -independent relaxations were assessed blindly in a Halpern arteriograph. A proximal segment of dissected right MCA was mounted in a small-vessel arteriograph (Living Systems Instrumentation) on 2 glass cannulas perfused with oxygenated Krebs' solution. Lumen diameter was measured by image analysis. Perfusion pressure was slowly increased to 75 mm Hg, and MCA was stabilized for 1 hour. To test endothelium-dependent relaxation, MCA was preconstricted by serotonin (10⁻⁶ mol/L), and when the diameter reached a steady state, a cumulative concentration (from 10⁻⁹ to 10⁻⁵ mol/L) of acetylcholine was added to the serotonin-containing Krebs' solution until a steady dilatation was attained. After washout, sodium nitroprusside (3×10⁻⁷ mol/L) was applied on serotonin-preconstricted MCA to test endothelium-independent vasorelaxation. Vasorelaxation was expressed as percentage of increase of the preconstricted artery diameter.

Experimental Protocol

In a first step, rtPA (alteplase, Boehringer Ingelheim) was administered to SHR at several doses: 3 mg/kg (n=13), 10 mg/kg (n=11), and 30 mg/kg (n=6) 6 hours after focal ischemia induction to test the dose-dependent effect of ICH occurrence. Doses of rtPA that were much higher than those used in humans were justified because the clot lysis system in rodents is less responsive to rtPA than the system in humans. Control animals (n=12) received an equivalent volume of isotonic normal saline. Saline or rtPA was administered with the use of an infusion pump over a period of 1 hour, with an initial bolus of 10% dose.

In a second step, saline, rtPA (10 mg/kg), or rtPA+TLP solution was administered 6 hours after focal ischemia induction in SHR (n=6, n=13, and n=25, respectively) to test the role of rtPA-induced TLP on ICH. MCA vasoreactivity was compared with control MCA of saline-treated sham WKY (n=7). Saline-treated sham SHR were not used as control because in this group, MCA response was characterized by contraction for the highest concentration of acetylcholine. Preliminary experiments showed that MCA reactivity impairment in ischemic SHR and WKY was similar (data not shown).

All experiments were performed in strict accordance with guidelines of the French National Institutes of Health and Department of Agriculture.

Statistical Analysis

All data were expressed as mean±SE. Continuous variables (mean arterial blood pressure, blood gases, infarct volumes, number of petechiae) were compared with 1-way ANOVA, followed by a post hoc protected least significant difference Fisher test analysis if variance analysis was significant. A χ² analysis was performed to compare results expressed as frequency. A value of P<0.05 was considered to indicate statistical significance.

Results

Dose-Dependent Effect of rtPA on Hemorrhagic Complications

Mortality

Fewer than 10% of animals died for technical reasons during or immediately after induction of cerebral ischemia, and they were not taken into account in the study. From the start of saline or rtPA administration until the time the rats were killed, mortality was 8% in the control group versus 36% in the various groups perfused with rtPA (P<0.001). Mortality was higher in rats receiving rtPA 30 mg/kg, although this remained statistically insignificant (Table 1).

ICH Incidence and Severity

All rats receiving the total 1-hour rtPA or saline treatment were considered in the analysis. No ICH was observed visually in the control group in contrast to groups receiving

<table>
<thead>
<tr>
<th>TABLE 1. Effects of rtPA Dose on Mortality and Intracerebral Hemorrhage Incidence and Severity in Ischemic SHR</th>
<th>NaCl 0.9%</th>
<th>rtPA 3 mg/kg</th>
<th>rtPA 10 mg/kg</th>
<th>rtPA 30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality, n (%)</td>
<td>1/12 (8)</td>
<td>4/13 (31*)</td>
<td>3/11 (27*)</td>
<td>3/6 (50*)</td>
</tr>
<tr>
<td>Incidence of hemorrhagic complications, n (%)</td>
<td>0/11 (0)</td>
<td>6/9 (66*)</td>
<td>5/8 (62*)</td>
<td>2/3 (66*)</td>
</tr>
<tr>
<td>Severity of hemorrhagic infarction (no. of petechiae)</td>
<td>0</td>
<td>2.4±1.1*</td>
<td>2.3±1.2*</td>
<td>1.7±0.9*</td>
</tr>
<tr>
<td>Corrected total infarct volume, mm³</td>
<td>149±9</td>
<td>135.7</td>
<td>147±13</td>
<td>146±21</td>
</tr>
</tbody>
</table>

*P<0.001 as compared with saline-treated ischemic SHR. Values are mean±SEM.
rtPA (total incidence of ICH: 64%; \( \frac{P}{H1021} 0.01 \)). Incidence and severity of ICH were independent of the rtPA dose (Table 1).

Parenchymal hematoma was observed in rtPA 3 mg/kg (n=1) and 10 mg/kg (n=1) groups.

**Infarct Volume**

No change in infarct volume was observed between the 3 rtPA-treated groups and the control group (Table 1). Physiological parameters remained similar before, during, and after ischemia between the different groups.

**Influence of rtPA and TLP on Hemorrhagic Complications**

**Mortality**

Mortality rate observed after administration of rtPA+TLP was insiginificantly higher than in the rtPA group (52% versus 23%; \( P=0.09 \)) (Table 2).

**ICH Incidence and Severity**

All rats that had received the total 1-hour treatment were included in the analysis. No difference in ICH incidence was observed between the rtPA+TLP group (84%) and the rtPA group (70%), while ICH severity was significantly more severe in the rtPA+TLP group (8.8±2.3 vs 3.0±1.0 petechiae in rtPA group; \( P<0.001 \)) (Table 2; Figure 1c).

Hematoma occurrence was similar in the 2 groups (n=1 and n=2, respectively).

**Stroke Volume**

Corrected total infarct volume was significantly increased in the rtPA+TLP group (182.47±9.67 versus 144.41±14.84 mm\(^3\) in rtPA group; \( P<0.01 \)) (Table 2; Figure 1d). No significant changes in physiological parameters were observed.

**Effect of rtPA and TLP on Vascular Reactivity**

The endothelium-dependent response was altered in saline-treated ischemic SHR, as demonstrated by a decrease in maximal relaxation (12±1%) in comparison to sham WKY (20±1%; \( P<0.0001 \)) (Figure 2). The impairment of MCA relaxation induced by ischemia in SHR was not significantly modified by rtPA administration. In contrast, MCA relaxing response to acetylcholine was significantly lowered in ischemic SHR receiving rtPA+TLP (\( P<0.05 \); Table 3, Figure 2). Contractile responses to serotonin were similar in the 3 groups as well as endothelium-independent relaxation (Table 3).

**Discussion**

Our study confirmed, in a mechanical model of MCA occlusion, that administration of rtPA, a fibrinolytic agent, provoked hemorrhagic complications. This effect was not dependent on dose or on greater infarct volume. In addition, ICH severity was increased by the administration of a solution resulting from in vitro thrombolysis induced by rtPA. This increased severity was associated with increased infarct volume and worsened MCA postischemic endothelium function. In contrast, no modification of MCA endothelium-independent function was observed.

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**Table 2. Effects of rtPA, Alone or in Combination With Thrombus Lysis Products, on Mortality and Intracerebral Hemorrhage Incidence and Severity in Ischemic SHR**

<table>
<thead>
<tr>
<th></th>
<th>rtPA 10 mg/kg</th>
<th>rtPA 10 mg/kg+TLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality, n (%)</td>
<td>3/13 (23)</td>
<td>13/25 (52)</td>
</tr>
<tr>
<td>Incidence of hemorrhagic complications, n (%)</td>
<td>7/10 (70)</td>
<td>10/12 (84)</td>
</tr>
<tr>
<td>Severity of hemorrhagic infarction (no. of petechiae)</td>
<td>3.0±1.0</td>
<td>8.8±2.3*</td>
</tr>
<tr>
<td>Corrected total infarct volume, mm(^3)+SEM</td>
<td>144±15</td>
<td>182±10†</td>
</tr>
</tbody>
</table>

*\( P<0.001 \) and †\( P<0.01 \) as compared with rtPA 10 mg/kg group. Values are mean±SEM.

TLP indicates thrombus lysis products.
Incidence and severity of rtPA-related hemorrhages were not dependent on rtPA dose in our model. These findings were in accordance with results obtained in some clinical trials, although other clinical trials tended to disprove this. Because the rtPA dose of 10 mg/kg presented a better compromise between mortality and hemorrhage occurrence, we used it for the second part of the experiments. We also observed the time dependency of ICH occurrence because no hemorrhage was observed when rtPA was perfused 3 hours after ischemia (data not shown). These results were congruent with conclusions of the meta-analysis of clinical trials, which recommended thrombolysis only in the 3 hours after stroke onset.

Hemorrhagic complications induced by rtPA have been described previously in thromboembolic models of cerebral ischemia. In our mechanical model, we found that hemorrhagic complications after rtPA occurred as frequently as described in thromboembolic models, but the hypothesis of thrombus involvement emerged because severity was surprisingly lower. Apart from differences in the profiles of blood flow, blood-brain barrier leakage, and metalloproteinase expression between mechanical and thromboembolic models, which contribute to explain ICH occurrence, TLP presence may affect hemorrhage severity, as shown in our model. Plasmin, generated by the interaction between rtPA and thrombus, in particular may play a crucial role. This hypothesis was supported by data of some experimental studies, in which plasmin in high doses led to hemorrhages. Moreover, in clinical studies, ICH incidence among patients treated with rtPA for myocardial infarction was correlated with a high plasma level of fibrinogen degradation products.

Increased ICH severity in the rtPA+TLP group was associated with significantly larger infarct volume. This could contribute to the mortality in this group. Moreover, large infarct volume was a risk factor for bleeding, particularly in the ischemic area. The influence of infarct volume may also be indirect and may be linked to greater vascular wall impairment, as suggested by the parallelism between neuroprotection and vascular protection in a pharmacological model of preconditioning.

The increase in postischemic vascular impairment observed in the rtPA+TLP group in parallel with the increase in hemorrhage severity supports the hypothesis of vascular involvement in ICH physiopathology. Indeed, plasmin, a serine protease that is able to induce disruption of the blood-brain barrier, has been linked to hemorrhagic complications in animal and human experiments. This plasmin-induced blood-brain barrier disruption was related to impairment of brain microvascular endothelium, loss of matrix integrity, fibrin deposition in vessel walls, and vascular contraction. The role of plasmin in ICH may be complementary to involvement of other serine proteases such as metalloproteinases, which were previously described as another mechanism of ICH occurrence.

These data confirm that rtPA perfusion 6 hours after induction of ischemia leads to ICH, in contrast to animals perfused with saline. In addition, these data suggest that rtPA-induced TLP, and in particular plasmin, rather than rtPA alone, contribute to the severity of ICH by mechanisms involving both infarct volume and postischemic endothelial function, while the relationship between the 3 phenomena remains to be demonstrated.

Acknowledgments

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