Effects of Recombinant Tissue Plasminogen Activator After Intraluminal Thread Occlusion in Mice
Role of Hemodynamic Alterations

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Background and Purpose—It has been suggested that recombinant tissue plasminogen activator (rtPA) may cause an aggravation of injury after transient focal ischemia via excitotoxic side effects. Such rtPA toxicity would be of major clinical significance since rtPA is increasingly used in stroke treatment. This study was conducted to evaluate the effects of dose, application time, and hemodynamic changes after intravenous rtPA treatment in focal ischemia.

Methods—Mice were subjected to a 90-minute episode of middle cerebral artery thread occlusion, and rtPA effects were assessed by laser-Doppler flowmetry, [14C]iodoantipyrine autoradiography, and triphenyltetrazolium chloride staining.

Results and Conclusions—We provide evidence that rtPA provokes complex hemodynamic alterations in the ischemic brain tissue, which include an initial hyperperfusion and a more delayed hypoperfusion response. Changes are most pronounced in the periphery of the ischemic infarct, where regional blood flow drops below critical thresholds of tissue viability. Our observations suggest that changes of perfusion may at least partly explain the rtPA-induced increase of infarct size, which has previously been reported and which we also confirmed in the present experiments. Notably, both the secondary hypoperfusion and increase of infarct volume were abolished when rtPA-treated animals received additional heparin infusions. This finding suggests that a secondary hypercoagulability may compromise brain perfusion after rtPA delivery. Accordingly, early treatment with heparin might help to prevent the rtPA-induced changes. (Stroke. 2001;32:2641-2647.)

Key Words: hemodynamics ■ heparin ■ ischemia, focal ■ plasminogen activator, tissue-type ■ thrombolysis ■ mice

The issue of whether recombinant tissue plasminogen activator (rtPA) may cause an aggravation of injury after transient focal ischemia has been the subject of controversial discussion.1 This discussion is based on experiments by Wang et al,2 which suggested that intravenous rtPA administration increases brain injury after reversible thread occlusion of the middle cerebral artery (MCA) in mice. On the basis of additional experiments showing that reversible MCA occlusion in tissue plasminogen activator (tPA)–deficient mice produces infarcts that are considerably smaller than those in wild-type animals,2 it was proposed that the increase of injury may be due to excitotoxic side effects of rtPA, possibly by enhancing N-methyl-D-aspartate receptor–mediated signaling.3 However, the observations by Wang et al2 were the subject of controversy because some investigators were unable to confirm their findings.4–8 For this reason, the effects of rtPA after brain ischemia remained largely unclear.9 Since there is a growing role of rtPA in clinical stroke treatment, we examined the effects of rtPA in a more detailed way. In the present experiments rtPA, either alone or in combination with heparin, was intravenously applied at various doses (0.2, 1, 2, or 10 mg/kg body wt) and at different time points before, during, or after a 90-minute episode of intraluminal thread occlusion of the MCA in mice. The degree of injury was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Furthermore, to rule out the possibility that the effects of rtPA might be related to hemodynamic changes, which was contrary to previous assumptions, cerebral laser-Doppler flow (LDF) was recorded during ischemia and up to 90 minutes after the onset of reperfusion, and changes of regional cerebral blood flow (CBF) were measured by [14C]4-iodo-N-methyl-antipyrine (IAP) autoradiography.

Materials and Methods

Experimental Groups
All experimental procedures were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals with approval of local government authorities. Adult male C57BL/6j mice weighing 21 to 28 g were assigned to the following groups: group A: 90 minutes of focal ischemia followed by intravenous infusion of 0.2 mL isotonic normal saline, starting at the onset of the reperfusion period (infusion time 30 minutes); groups B to E: 90 minutes of ischemia followed by intravenous infusion of 0.2 mL carrier solution containing 0.2, 1, 2, or 10 mg/kg body wt rtPA

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Briefly, 0.2 μCi \([^{14}\text{C}]\text{IAP}\) (specific activity, 40 to 60 mCi/mmol; Amersham Life Sciences) dissolved in 0.2 mL 0.9% saline was injected intraperitoneally. Two minutes later, animals were decapitated, and arterial blood samples (50 to 100 μL) were taken from the animals’ trunks and subsequently measured in a liquid scintillation counter (Wallac 1410, Pharmacia). Brains were quickly removed and frozen with dry ice. Twenty-micrometer coronal sections were prepared at –20°C on a cryostat, dried on a hot plate, and exposed to x-ray film (Amersham Hyperfilm) with calibrated \([^{14}\text{C}]\) standards. \([^{14}\text{C}]\) autoradiograms and standards were digitized with a charge-coupled device camera connected to an image processing system (ImageMG; National Institutes of Health), and local \([^{14}\text{C}]\) radioactivity was determined by quantitative autoradiography. Local CBF was calculated according to the algorithm described by Sakurada et al., as follows:

\[
[C(t)] = \lambda K_i \int_0^T C(t)e^{-K_i t} dt
\]

where \(K_i\) is equal to \((f_i m)/(f_i V)\), \(\lambda\), \(K_i\) can be rearranged with \(F_i\) with the IAP diffusion coefficient \(m\) equal to 1; \(F_i\) is the flow rate \(f_i\) (milliliters per minute) divided by the tissue weight \(V\) (grams); and \(\lambda\) is the tissue blood partition coefficient for IAP equal to 0.8, which cancels. Therefore, the ratio of local tissue radioactivity \(C_i(t)/T\) to \(F_i\) equals the flow-related convolution integral, which is dependent on the time course of blood radioactivity, as follows:

\[
\frac{C_i(t)}{F_i} = \int_0^T C_i(t)e^{-K_i t} dt
\]

Convolution integrals for various blood flow rates were calculated from trunk blood sampling by assuming linearity of \([^{14}\text{C}]\text{IAP}\) radioactivity between the intraperitoneal \([^{14}\text{C}]\text{IAP}\) injection and the termination of the experiment, as has previously been shown.\(^{14}\)

Statistical Analysis

For statistical data comparison, a standard software package (SPSS for Windows) was used. Differences between groups in LDF recordings were calculated by repeated-measures ANOVA. Since the number of LDF time points exceeded the number of animals per group, repeated-measures statistics were performed on average numbers of LDF time points. Differences between single LDF values were determined by unpaired \(t\) tests. Differences between groups in infarct volume and brain edema were calculated by 1-factor ANOVA followed by least significant difference tests. Differences in regional CBF between saline- and rtPA-treated animals as well as rtPA- and rtPA/heparin-treated animals were calculated by ANOVA as well as unpaired \(t\) tests. All values are given as mean±SEM. Values of \(P<0.05\) are considered to indicate statistical significance.

Results

Dose-Dependent Effects of rtPA During Postischemic Reperfusion

Animals were treated either with isotonic saline (group A) or with rtPA at various doses (0.2, 1, 2, or 10 mg/kg body wt; groups B to E) immediately after the onset of reperfusion, ie, retraction of the thread, and the degree of injury was assessed by TTC staining 24 hours later.

Laser-Doppler Flowmetry

In normal saline-treated control animals, thread insertion resulted in a reproducible decrease of LDF to values of 16.1±3.4% of control, which remained constant throughout the 90-minute episode of MCA occlusion (Figure 1). Thread retraction resulted in a restoration of LDF to values of 20.1±2.1% above baseline.
94.3±12.2% of control, followed by a mild secondary LDF decline to 71.9±7.5% within 90 minutes after the onset of reperfusion (Figure 1A). In animals treated with rtPA at doses of 0.2, 1, or 2 mg/kg body wt rtPA (groups B to E), animals treated with 2 or 10 mg/kg rtPA in combination with intravenous heparin (200 IE/kg) (groups F and G), and animals treated with heparin alone (200 IE/kg) (group H) are shown. Note the initial hyperperfusion response, which was detected in animals treated with 10 mg/kg rtPA (group E) but not in animals treated with lower rtPA doses (groups B to D). Further note the secondary LDF decrease, which is more pronounced in rtPA-treated animals (groups B to E) than in normal saline-treated controls (group A). Because of the secondary decrease of blood flow, LDF values drop significantly below control levels after 90 minutes of reperfusion in animals treated with 1 and 2 mg/kg rtPA (groups C and D). The secondary LDF decrease in rtPA-treated animals is markedly attenuated by additional treatment with heparin. Heparin alone, on the other hand, does not have an effect on postischemic LDF recordings. *Significantly different from normal saline-treated control animals (group A; P<0.05); §significantly different from rtPA-treated animals (groups D and E; P<0.05).
Effects of rtPA Administration During Ischemia

Animals were treated with rtPA (0.2 or 2 mg/kg body wt; groups I to J) during ischemia, ie, starting 15 minutes after thread insertion, and brain injury was examined 24 hours after the onset of reperfusion.

Laser-Doppler Flowmetry

In animals treated with rtPA during ischemia, LDF recordings during thread occlusion did not differ from those of control animals (11.2±2.2% and 14.2±2.7% of preischemic values after rtPA doses of 0.2 and 2 mg/kg; Figure 2, groups I and J). On the other hand, there was a trend toward an LDF increase in these animals after thread retraction (118.4±14.2% and 118.5±11.2%, respectively, of preischemic values; Figure 2I and 2J), which did not reach statistical significance. Measurements remained mildly elevated throughout the LDF recording period (93.5±20.0% and 89.5±8.4% even after 90 minutes of reperfusion).

Ischemic Injury and Brain Edema

A slight but nonsignificant reduction of the infarct volume was noted in animals treated with rtPA during ischemia, independent of the rtPA dose applied (27.1±6.7 and 28.2±5.0 mm³, respectively; Figure 3). Our data are in accord with previous results of our group,4 indicating that rtPA administration during ischemia reduces rather than increases the degree of injury. The degree of brain swelling was not influenced in animals receiving rtPA during ischemia compared with saline-treated control animals (Figure 3).

Effects of rtPA Administration Before Ischemia

Animals were treated with rtPA (0.2 or 2 mg/kg body wt; groups K and L) before ischemia, ie, 45 minutes before insertion of the thread, and tissue injury was assessed 24 hours after the onset of reperfusion.

Laser-Doppler Flowmetry

In animals treated with rtPA before ischemia, LDF values during thread occlusion were significantly reduced (LDF decrease to 7.6±0.9% and 6.8±1.2% of preischemic control; P<.05) compared with saline-treated control animals (Figure 2, groups K and L). Postischemic LDF values, on the other hand, did not differ from those of control animals (Figure 2K and 2L).

Ischemic Injury and Brain Edema

The infarct volume was not influenced in animals treated with 0.2 mg/kg rtPA (43.2±8.6 mm³) compared with control animals. On the other hand, the infarct size was significantly elevated in animals receiving 2 mg/kg body wt rtPA before induction of ischemia (72.3±3.9 mm³; P<0.01; Figure 3), in accord with the aggravation of ischemia. Brain edema was not changed in animals receiving rtPA before ischemia (Figure 3).
Effects of rtPA and rtPA Plus Heparin in Nonischemic Control Animals

Additional control animals, not submitted to focal ischemia, were treated with isotonic saline, rtPA (10 mg/kg body wt), or rtPA in combination with heparin (200 IE/kg), and LDF recordings were performed over 90 minutes. In these animals, neither rtPA nor rtPA plus heparin had significant effects on LDF measurements (Table).

Regional CBF Autoradiography With [14C]IAP

To corroborate our observation that intravenous rtPA may cause a secondary hypoperfusion of the ischemic brain tissue, which may be responsible for the aggravation of injury, additional animals were treated with either isotonic saline, rtPA (10 mg/kg body wt), or an identical dose of rtPA plus heparin (200 IE/kg) immediately after thread retraction and submitted to regional CBF autoradiography 2 hours after the onset of reperfusion.

In the cingulate cortex, which was studied as a reference region outside the MCA territory, neither rtPA nor rtPA in combination with heparin had an influence on regional CBF (Figure 4). In the penumbral sensory cortex, on the other hand, intravenous rtPA significantly reduced regional CBF from 78.9 ± 2.3 to 33.5 ± 11.3 mL/100 g per minute (Figure 4). Notably, this reduction of blood flow was completely reversed and even shifted above control levels after additional heparin treatment (regional CBF, 137.2 ± 38.4 mL/100 g per minute; Figure 4). Similarly, there was a trend toward a reduction of regional CBF after intravenous rtPA and an amelioration of blood flow after rtPA plus heparin in the infarcting parietal cortex and caudate putamen (Figure 4). However, these effects did not reach statistical significance.

Discussion

In the present study we examined the effects of intravenous rtPA administered at various doses (0.2, 1, 2, or 10 mg/kg body wt) and time points before, during, and after a 90-minute episode of intraluminal thread occlusion of the MCA in mice. Regional CBF was recorded during ischemia and up to 90 minutes after the onset of reperfusion by LDF and [14C]IAP autoradiography and correlated with the degree of injury, as determined by TTC staining.

Controversial data have been obtained in the past concerning the effects of rtPA after focal brain ischemia. It has been suggested that rtPA may cause an aggravation of injury after transient focal ischemia in mice, possibly due to toxic side effects of rtPA. This hypothesis was based on experiments showing that intravenous administration of 0.9 mg/kg rtPA increases the infarct size 24 hours after a 2-hour episode of MCA occlusion. These results were supported by additional studies showing that transient MCA occlusion in tPA-deficient mice produces infarcts, which are only approximately 50% as large as those in wild-type animals. However, the results by Wang et al were considered controversial because subsequent investigators were unable to confirm that rtPA increases brain injury after reversible MCA thread occlusion. One group even showed that brain damage may not be reduced but rather exacerbated under certain conditions of focal ischemia when tPA-deficient mice are compared with wild-type animals on a matched genetic background. Although there has been increasing evidence from models of excitotoxic brain injury that rtPA may facilitate...
neuronal death,\textsuperscript{3,9,16,17} the effects of rtPA remained largely unclear.

The present report demonstrates that rtPA causes a dose-dependent increase of ischemic injury, which is in close agreement with previous findings by Wang et al.\textsuperscript{2} In contrast to those previous data, however, the present results do not necessarily support the conclusion that the increase of injury may be attributed to excitotoxic side effects, although toxic effects of rtPA have previously been described in cell culture.\textsuperscript{18–20} Conversely, the present report shows that rtPA infusion is followed by complex hemodynamic changes in the ischemic brain tissue, which might outweigh such toxic effects and need to be taken into account in the present discussion. After high (ie, 10 mg/kg body wt) but not after lower (up to 2 mg/kg body wt) doses of rtPA, we observed an initial hyperperfusion response immediately after the onset of reperfusion. Although this dose is well above those doses usually applied in humans (0.9 mg/kg body wt),\textsuperscript{21} it much better reflects the pathophysiological situation because the thrombolytic activity of rtPA is considerably lower in rodents than in human patients. In fact, it has been shown that because of species differences, an approximately 10-fold higher rtPA dose is required in mice to achieve activation of the plasmin system comparable to that in humans.\textsuperscript{22,23} Hence, an rtPA dose of 10 mg/kg body wt in rodents almost equals the dose of 0.9 mg/kg body wt in humans.\textsuperscript{21} Thus, our data suggest that the hyperperfusion may be a consequence of the direct fibrinolytic action of rtPA and may be explained by the prevention of spontaneous clot formation after thread retraction.

However, the amelioration of blood flow was only short-lasting and was followed by a secondary decrease of Doppler flow, which dropped below levels of control animals within 90 minutes after thread retraction in rtPA-treated animals. Further autoradiographic experiments showed that this secondary hypoperfusion was most pronounced in the ischemic border zone (ie, the sensory cortex), where regional CBF decreased to 33.5±11.3 mL/100 g per minute within 2 hours after reperfusion. Since regional CBF decreased below the critical flow threshold required for tissue survival, which is between 50 and 55 mL/100 g per minute,\textsuperscript{24} our results strongly suggest that the reduction of blood flow was at least partly responsible for the rtPA-induced increase in infarct size. The fact that both hyperperfusion and hypoperfusion responses were noted after rtPA delivery may explain why the dose-response dependency between rtPA and infarct size was not linear in the present study but rather followed a bell-shaped relationship. In our study the maximum increase in infarct size was observed at rtPA doses between 1 and 2 mg/kg body wt, ie, at doses at which the secondary hypoperfusion response became prominent. On the other hand, the increase in infarct size was less pronounced at higher rtPA doses, ie, at 10 mg/kg body wt, which may due to the fact that the now detected hyperperfusion response opposed the injury-aggravating effect.

Our data thus raise a question regarding the reasons for the hemodynamic changes after rtPA treatment. The biological half-life of rtPA is very short (8 to 12 minutes).\textsuperscript{25} It is therefore unlikely that rtPA effects were evoked by rtPA itself. Moreover, the aggravation of brain injury in this study was not associated with an increase of brain swelling, suggesting that cytotoxic edema was not responsible for the secondary hypoperfusion. A more likely explanation for the secondary CBF decrease may be that rtPA treatment resulted in a secondary shift in the balance between coagulation and anticoagulation. This idea is supported by the observation of the present study that intravenous treatment with heparin completely abolished both the decrease of blood flow and increase in infarct size after rtPA infusion. In fact, it has previously been demonstrated that rtPA treatment may cause an activation of coagulation cascades after stroke.\textsuperscript{26} Additionally, it has been shown that administration of rtPA may lead to the activation of plasminogen activator inhibitor-1, thereby also resulting in inhibition of endogenous fibrinolysis.\textsuperscript{27}

It is noteworthy that the effects of rtPA depended on the application time of rtPA. In contrast to animals treated during reperfusion, a slight but nonsignificant reduction of infarct size was noted in animals receiving rtPA infusions during MCA occlusion. In these animals, posts ischemic LDF was slightly improved, which may be explained by the prevention of clot formation at the tip of the occlusion thread. Our data confirm previous findings from our group showing that application of rtPA during thread occlusion causes a reduction rather than aggravation of brain injury.\textsuperscript{4} On the other hand, we noticed a dose-dependent increase of the infarct volume in animals treated with rtPA before ischemia. It is noteworthy that the aggravation of injury was even more pronounced in these animals than when rtPA was given during reperfusion. In animals treated with rtPA before ischemia, there were no changes of posts ischemic LDF measurements, but blood flow values during thread occlusion were significantly reduced compared with those of control animals. The increase of brain injury may therefore be a consequence of the more severe ischemia in these animals.

In summary, the present report demonstrates that the aggravation of injury previously reported after transient focal ischemia\textsuperscript{2} depends on both the rtPA dose and application time. Our results show that intravenous rtPA infusion is followed by complex hemodynamic changes in the ischemic tissue, involving both initial hyperperfusion and secondary hypoperfusion responses. According to our data, the secondary hypoperfusion reaches critical flow thresholds in the border zone of the ischemic territory (ie, the sensory cortex), which indicates that these hemodynamic disturbances might be responsible at least in part for the increase of infarct size. Since both the reduction of blood flow and the increase of infarct volume may be reversed by additional heparin treatment, our data suggest that an early combination therapy of intravenous rtPA with heparin may be useful in preventing such secondary changes. Among clinical neurologists, there is a controversial discussion about whether rtPA should be combined with heparin after intravenous thrombolysis.\textsuperscript{28,29} The present data argue in favor of a combination strategy of rtPA and heparin, although care must be taken not to increase the bleeding risk after stroke.

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