Postischemic (6-Hour) Treatment With Recombinant Human Tissue Plasminogen Activator and Proteasome Inhibitor PS-519 Reduces Infarction in a Rat Model of Embolic Focal Cerebral Ischemia

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Background and Purpose—The proteasome inhibitor PS-519 blocks activation of nuclear factor-κB, a major mediator of inflammation. We tested the hypothesis that combination treatment of recombinant human tissue plasminogen activator (rhtPA) and PS-519 extends the therapeutic window for treatment of stroke with rhtPA without increasing incidence of hemorrhagic transformation.

Methods—The middle cerebral artery (MCA) of male Wistar rats (n=56) was occluded by an embolus. After embolization, animals were randomly divided into the following groups: PS-519 treatment groups: PS-519 was given at 2, 4, or 6 hours after MCA occlusion; rhtPA treatment groups: rhtPA was given at 2 or 4 hours after MCA occlusion; combination treatment groups: PS-519 and rhtPA were given at 2, 4, or 6 hours after MCA occlusion; control group: the same volume of saline was given at 2 hours after MCA occlusion.

Results—Administration of PS-519 alone at 2 or 4 hours, but not 6 hours, significantly (P<0.05) reduced infarct volume and improved neurological recovery compared with the control group. Administration of rhtPA alone at 2 hours, but not 4 hours, significantly (P<0.05) reduced infarct volume and improved neurological recovery compared with the control group. Furthermore, combination treatment with rhtPA and PS-519 even at 6 hours significantly (P<0.05) reduced infarct volume, improved neurological recovery, and did not increase the incidence of hemorrhagic transformation compared with the control group or the group treated with PS-519 alone.

Conclusions—Our data suggest that combination treatment with PS-519 and rhtPA extends the neuroprotective effect to at least 6 hours after embolization. (Stroke. 2001;32:2926-2931.)

Key Words: inflammation ■ middle cerebral artery occlusion ■ tissue plasminogen activator ■ rats

Thrombolysis with tissue plasminogen activator (tPA) within 3 hours of the onset of ischemic stroke improves clinical outcome. However, the therapeutic time window is narrow, and administration of tPA to stroke patients beyond 3 hours increases the likelihood of hemorrhagic transformation and fails to provide any therapeutic benefit.1,2 Adverse effects induced by delayed treatment with tPA may be associated with endothelial dysfunction and injury resulting from interaction of the inflammatory cell with the endothelial cell.3,4 In support of this hypothesis, an adjuvant therapy with antibodies against adhesion molecules and tPA results in reduction of infarction volume and neutrophil infiltration and improves functional neurological recovery in animal models of embolic middle cerebral artery (MCA) occlusion.5 Furthermore, administration of recombinant human tPA (rhtPA) at 4 hours after embolic MCA occlusion significantly increases expression of P-selectin, E-selectin, and intercellular adhesion molecule-1 (ICAM-1) in ischemic brain.6

Inflammatory response promotes ischemic cell damage after stroke. The inflammatory mediators such as adhesion molecules and cytokines play an important role in the inflammatory cascade.7 Adhesion molecules regulate the leukocyte-endothelial interactions.8 Activation of adhesion molecules facilitates leukocyte rolling. Leukocytes then firmly adhere to endothelia, infiltrate the ischemic lesion, and consequently induce ischemic brain damage. Cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) mediate the inflammatory responses by upregulating the adhesion molecules such as ICAM-1 and E-selectin.9-11 Since the inflammatory response typically develops hours or days after ischemic insult, inflammatory mediators such as adhesion molecules and proinflammatory...
cytokines participating in this secondary inflammatory cascade are potential therapeutic targets for brain ischemia.

Nuclear factor kappa B (NF-κB) is a protein transcription factor. In the resting state, NF-κB is sequestered in the cytoplasm by the inhibitory protein IκB, which prevents its translocation to the nucleus. In response to various pathogenic stimuli, specific kinases phosphorylate IκB, leading to its proteolysis and dissociation from NF-κB. The activated NF-κB travels to the nucleus, where it binds to the promoter of a large number of genes, including cytokines and adhesion molecules such as interleukin-1β (IL-1β), IL-6, TNF-α, E-selectin, and ICAM-1, suggesting that NF-κB activation is an upstream common pathway involved in the upregulation of multiple adhesion molecules and cytokines. Occlusion of the MCA induces upregulation of NF-κB in ischemic brain.

Proteasome inhibitors block activation of NF-κB by preventing the degradation of IκB. In vitro, proteasome inhibitors prevent TNF-α-mediated induction of adhesion molecules. Administration of a potent proteasome inhibitor, PS-519, to ischemic rats significantly reduces infarction and attenuates leukocyte infiltration in ischemic brain. Given the likely role of inflammation limiting the therapeutic window for tPA, a potential strategy to extend the therapeutic window of thrombolysis is to block NF-κB activation. Accordingly, in the present study we evaluated the effect of the proteasome inhibitor PS-519 in combination with rhtPA in a rat model of focal cerebral embolic ischemia. We demonstrate that coadministration of PS-519 and rhtPA at 6 hours after the onset of stroke significantly reduces infarct volume, improves neurological recovery, and does not increase the incidence of hemorrhagic transformation.

Materials and Methods

All experimental procedures were approved by the Care of Experimental Animals Committee of Henry Ford Hospital.

General Procedures

Male Wistar rats (n = 56) weighing 320 to 380 g were used in the present study. Rats were anesthetized with 3.5% halothane and maintained with 1.0% to 2.0% halothane in 70% N2O and 30% O2 with the use of a face mask. Rectal temperature was maintained at 37°C throughout the surgical procedure with a feedback-regulated water heating system. The right femoral artery and vein were cannulated for measuring physiological parameters, blood pressure, mean arterial blood pressure, and drug administration.

Preparation of the Embolus

Femoral arterial blood from a donor rat was withdrawn into 20 cm of polyethylene tubing (PE-50) and retained in the tube for 2 hours to clot at room temperature and subsequently retained for 22 hours at 4°C. Four centimeters of the PE-50 tube containing clot was cut and attached at each end to a 40-mm PE-10 tube interconnected by a syringe filled with saline. The clot was shifted by continuous alternating movement from one syringe to the other for 5 minutes. A single clot (~1 μL) was transferred to a modified PE-50 catheter with a 0.3-mm outer diameter filled with saline.

Animal Model

The MCA was occluded by placement of an embolus at the origin of the MCA. Briefly, under an operating microscope (Carl Zeiss, Inc), the right common carotid arteries, right external carotid artery (ECA), and internal carotid artery (ICA) were isolated via a midline incision. A modified PE-50 catheter with a 0.3-mm outer diameter filled with a single clot, which was attached to a 100-μL Hamilton syringe filled with 0.9% saline, was introduced into the ECA lumen through a small puncture. A 15- to 16-mm length of catheter was gently advanced from the ECA into the lumen of the ICA. The clot in the catheter was injected into the ICA along with 2 to 3 μL of 0.9% saline. The catheter was withdrawn from the right ECA 5 minutes after injection. The right ECA was ligated.

Experimental Protocols

PS-519, the proteasome inhibitor (Millennium Pharmaceuticals Inc), was intravenously infused at a dose of 1.0 mg/kg, rhtPA (Genentech) was infused intravenously at a dose of 10 mg/kg as a 10% bolus, and the remainder was infused continuously over a 30-minute interval with a Harvard pump (Harvard Apparatus). Doses of rhtPA and PS-519 were used on the basis of previously published studies on rats. After embolization, animals were randomly divided into the following groups: to examine the effect of PS-519 alone on ischemia, PS-519 was administered to ischemic rats at 2 hours (n = 6), 4 hours (n = 6), or 6 hours (n = 6) after MCA occlusion; to examine the effect of rhtPA alone on ischemia, rhtPA was administered to ischemic rats at 2 hours (n = 6) or 4 hours (n = 6) after MCA occlusion; to examine the effect of combination therapy of rhtPA and PS-519 on ischemia, PS-519 and then rhtPA were administered at 2 hours (n = 6), 4 hours (n = 6), or 6 hours (n = 6) after MCA occlusion. The control group consisted of ischemic rats (n = 8) administered the same volume of 0.9% of saline at 2 hours after MCA occlusion.

Neurological Deficit

Neurological deficits were examined at 1, 2, and 7 days after MCA occlusion. A 4-point neurological score was used: 0, no deficit; 1, failure to extend the left forepaw fully; 2, circling to the left; 3, falling to the left; and 4, no spontaneous walking with a depressed level of consciousness.

Body Weight Loss

Animals were weighed before and 24, 48, and 168 hours after embolic ischemia. Body weight loss is presented as a percentage of preischemic body weight.

Histopathological Studies

All the animals were anesthetized with ketamine (44 mg/kg IM) and xylazine (13 mg/kg IM) and killed at 7 days after MCA occlusion. Each rat was transcardially perfused with heparinized saline followed by 10% formalin. The brain was removed from the skull and cut into 7 coronal blocks, each with 2-mm thickness. The brain tissue was processed and embedded, and 6-μm-thick paraffin sections from each block were cut and stained with hematoxylin and eosin (H&E) for evaluation of ischemic cell damage. Lesion volume was measured with the use of a Global Laboratory Image analysis program (Data Translation). The area of both hemispheres and the area containing the ischemic neuronal damage (mm2) were calculated by tracing the area on the computer screen. The lesion volume (mm3) was determined by multiplying the appropriate area by the section interval thickness. To reduce errors associated with processing of tissue for histological analysis, the ischemic volume is presented as the percentage of infarct volume of the contralateral hemisphere (indirect volume calculation).

Measurement of Hemorrhage

Gross hemorrhage, defined as blood evident to the unaided eye on the H&E-stained coronal sections, was evaluated on 7 H&E-stained coronal sections for each animal. Gross hemorrhagic rate is presented as the percentage of gross hemorrhage relative to the number of animals in each experimental group. Petechial hemorrhage, defined as a cluster of red blood cells outside of the lumen of blood vessels, was measured on 7 H&E-stained coronal sections with a Global Laboratory Image analysis program (Data Translation). Each H&E-stained coronal section was evaluated, and the distribution of hemorrhage was recorded under a ×40 objective. The area of hemorrhage (μm2) was calculated by tracing the areas of the...
computer screen, and a summation of the area of hemorrhage from each section was presented as the total area of hemorrhage.

Measurement of Brain Myeloperoxidase
Inflammatory cells within the brain were measured immunohistochemically with the use of an antibody against myeloperoxidase in all rats. A 6-μm-thick paraffin-embedded coronal section from the center of the ischemic lesion at the level of the anterior commissure (coordinates: interaural, 8.2 mm; bregma, 0.8 mm) was cut and stained with a polyclonal rabbit anti-human myeloperoxidase antibody (1:200 dilution; DAKO) for evaluation of neutrophils. Numbers of myeloperoxidase-immunoreactive cells were counted throughout the whole hemisphere at ×400 magnification. Only morphologically intact myeloperoxidase-immunoreactive cells with multilobed nucleus (morphologically typical neutrophils) were included in the counts. An adjacent H&E-stained section was used for measurement of the infarct area. Data are presented as the number of myeloperoxidase-positive cells relative to the infarct area (mm²).

Statistical Analysis
All values are presented as mean±SD. We first tested the normality and equal variances of each measure of interest. Data transformation tests were considered if data were ill behaved. A 1-way ANOVA was conducted to test the treatment effect on each outcome followed by least squares pairwise comparisons between control and PS-519 groups with and without rhtPA. A treatment effect was detected at P<0.05.

Results

Physiological Parameters and Mean Arterial Blood Pressures
There were no significant differences in arterial pH, Po₂, PcO₂, and mean arterial blood pressure among the experimental groups before ischemia and 1 hour after drug administration. All values of these parameters are within the physiological range.

Neurological Deficits
One hour after MCA occlusion, all rats exhibited neurological deficits (Table 1). Treatment with PS-519 at 2 hours or 4 hours after MCA occlusion significantly improved neurological recovery from 1 day to 7 days after ischemia compared with control animals. However, no significant difference in neurological score was detected between the 6-hour treated group and the control group at all time points. Likewise, animals treated with rhtPA at 2 hours after MCA occlusion exhibited a significant reduction in neurological deficits from the control group at 1, 2, and 7 days after ischemia. However, no significant difference in the neurological score was detected between the 4-hour rhtPA treatment group and the control group. In the combination treatment groups, neurological scores were significantly improved compared with control at 2 hours after MCA occlusion. The 4-hour treatment group showed significant improvement in neurological score at 2 and 7 days after MCA occlusion. The 6-hour treatment group showed a significant improvement in neurological function at 7 days after MCA occlusion.

Body Weight
Treatment with PS-519 at 2 or 4 hours but not 6 hours after MCA occlusion resulted in a significant (P<0.05) reduction of animal body weight loss at 7 days after MCA occlusion compared with the control animals (Figure 1). Treatment with rhtPA at 2 hours but not 4 hours after MCA occlusion significantly reduced body weight loss at 7 days after ischemia (Figure 1). However, the animals treated with combination of rhtPA and PS-519 at 2, 4, and even 6 hours after MCA occlusion exhibited a significant (P<0.05) reduction of body weight loss at 7 days after ischemia compared with the control group (Figure 1).

Infarct Volume
Treatment with PS-519 at 2 and 4 hours but not 6 hours after ischemia significantly (P<0.05) reduced infarct volume compared with the control group (Table 2). Treatment with rhtPA at 2 hours but not 4 hours after ischemia significantly (P<0.05) reduced infarct volume compared with the control group (Table 2). However, combination treatment with PS-519 and rhtPA at 2, 4, or even 6 hours after ischemia significantly reduced infarct volume compared with control or the 6-hour PS-519 alone group (Table 2).

Hemorrhage
Gross hemorrhage in the ipsilateral lesion was detected in 1 of 8 rats in the control group, 1 of 6 in the 2-hour rhtPA treatment group, and 2 of 6 in the 4-hour rhtPA treatment group (Figure 2). None of the rats treated with PS-519 alone and the rats treated with combination drugs had gross hemorrhage within the infarct area. Table 2 shows the total microscopic hemorrhage areas of all groups. Treatment with rhtPA at 4 hours after ischemia significantly (P<0.05)
increased areas with microscopic hemorrhage at 7 days after MCA occlusion (Table 2).

**Myeloperoxidase-Immunoreactive Cells**

Figure 3 shows that administration of PS-519 alone and in combination with rhtPA significantly reduced the density of myeloperoxidase-immunoreactive cells in the ischemic lesion at 2, 4, and 6 hours compared with the control group.

**Discussion**

The present study demonstrates that administration of the proteasome inhibitor PS-519 alone at 2 or 4 hours, but not 6 hours, after embolic stroke significantly reduced infarct volume and improved functional neurological recovery compared with the control group. Furthermore, coadministration of rhtPA and PS-519 even at 6 hours after embolic stroke significantly reduced infarct volume, improved functional neurological recovery, and did not increase the incidence of hemorrhagic transformation compared with the control group or the group treated with PS-519 alone. Thus, our data indicate that the inhibition of NF-κB activation with proteasome inhibitor is effective for treatment of acute (4-hour) stroke, and the combination of proteasome inhibitor and thrombolytic therapy increases the therapeutic window for rhtPA treatment to at least 6 hours after stroke.

Our data suggest that the therapeutic window for PS-519 at the current dose level is <6 hours after embolic focal cerebral ischemia in rats, which is consistent with previous data that administration of PS-519 at 2 and 4 hours after transient focal cerebral ischemia significantly reduced cerebral infarction. Failure of neuroprotection for PS-519 at 6 hours after ischemia may at least partly be caused by progressive cerebral microcirculatory impairments after MCA occlusion in which PS-519 cannot reach ischemic tissue. In support of this premise, the neuroprotective effect was achieved by combination treatment with PS-519 and rhtPA at 6 hours after ischemia compared with PS-519 treatment alone. However, treatment with rhtPA alone was effective in reducing infarct volume at 2 but not 4 hours after ischemia, which is consistent with previous findings. Collectively, these data indicate that combination treatment with PS-519 and rhtPA extends the window of the efficacy of rhtPA in the rat, supporting the hypothesis that to extend the therapeutic window for treatment of stroke, it is necessary to combine a thrombolytic treatment with an anti-inflammatory or a neuroprotective compound.

The mechanisms underlying the neuroprotective effect of PS-519 observed in the present study may be attributed to an upstream reduction in the cascade of inflammation after stroke. NF-κB controls expression of several genes for adhesion molecules and cytokines critically involved in inflammatory function. In vitro, under reoxygenation of hypoxic human brain endothelial cells, NF-κB is activated and is correlated with the upregulation of the extracellular adhesion molecule ICAM-1. Specific inhibition of NF-κB by gene transfer of 1κB-α suppressed the expression of cytokines in different cells lines. In vivo, activation of NF-κB-mediated inflammatory responses has also been

**TABLE 2. Absolute Hemisphere Volumes, Infarct Volumes, and Total Microscopic Hemorrhage Areas**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Contralateral Hemisphere Volume, mm³</th>
<th>Ipsilateral Hemisphere Volume, mm³</th>
<th>Infarct Volumes, mm³</th>
<th>Total Microscopic Hemorrhagic Areas, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Direct</td>
<td>Indirect</td>
</tr>
<tr>
<td>Control</td>
<td>670.5±59.6</td>
<td>605.5±42.1</td>
<td>234.1±78.2</td>
<td>169.8±51.3</td>
</tr>
<tr>
<td>PS-519 (2 h)</td>
<td>680.1±74.7</td>
<td>637.8±78.5</td>
<td>126.8±46.3*</td>
<td>84.4±36.0*</td>
</tr>
<tr>
<td>PS-519 (4 h)</td>
<td>696.5±47.3</td>
<td>649.6±42.4</td>
<td>151.0±57.7*</td>
<td>106.0±42.5*</td>
</tr>
<tr>
<td>PS-519 (6 h)</td>
<td>711.9±71.8</td>
<td>643.1±56.9</td>
<td>250.2±56.9</td>
<td>171.6±34.7*</td>
</tr>
<tr>
<td>rhtPA (2 h)</td>
<td>673.6±14.4</td>
<td>640.7±22.7</td>
<td>128.8±58.3*</td>
<td>96.2±40.6*</td>
</tr>
<tr>
<td>rhtPA (4 h)</td>
<td>711.5±43.0</td>
<td>638.0±39.0</td>
<td>267.7±26.4</td>
<td>194.4±21.1*</td>
</tr>
<tr>
<td>PS-519+rhtPA (2 h)</td>
<td>661.2±36.6</td>
<td>632.6±41.6</td>
<td>118.2±70.9*</td>
<td>98.6±61.4*</td>
</tr>
<tr>
<td>PS-519+rhtPA (4 h)</td>
<td>672.2±45.5</td>
<td>615.7±55.6</td>
<td>141.7±59.1*</td>
<td>103.3±41.9*</td>
</tr>
<tr>
<td>PS-519+rhtPA (6 h)</td>
<td>665.8±68.1</td>
<td>629.6±68.2</td>
<td>135.4±59.0*</td>
<td>109.0±42.8*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*P<0.05 vs control group.
shown in several animal models. PS-519 effectively attenuates inflammatory response in inflammation-related diseases. In a mouse model of asthma, administration of PS-519 into the lungs significantly reduced leukocyte numbers. Cardioprotective effects of PS-519 are associated with reduction of leukocyte accumulation and attenuated P-selectin surface expression on coronary vascular endothelium in a model of rat myocardial ischemia/reperfusion. The present study shows that PS-519 significantly reduced the myeloperoxidase-immunoreactive cells in the ischemic areas in all treatment groups. Myeloperoxidase is a marker for inflammatory cells, primarily neutrophils. Although monocytes also possess myeloperoxidase activity, monocytes contain much lower levels of myeloperoxidase (0.9% of cell weight) than neutrophils (2% to 5% of cell weight). In the present study each myeloperoxidase-immunoreactive cell had a multilobed nucleus, which is morphologically typical of neutrophils, while morphologically typical macrophages did not exhibit myeloperoxidase immunoreactivity. Therefore, the reduction of numbers of myeloperoxidase-immunoreactive cells represents a decrease in numbers of neutrophils. Our results are consistent with our previous finding that the measurement of myeloperoxidase-immunoreactive cells in the ischemic brain is a reliable method for quantifying neutrophil infiltration. Thus, the reduction of myeloperoxidase-immunoreactive cells indicates that administration of PS-519 significantly reduces neutrophil infiltration into the ischemic lesion, which is consistent with previous findings. Since certain adhesion molecules are target genes for NF-κB, PS-519 might inhibit adhesion molecule expression and thereby might reduce neutrophil infiltration. Thus, the neuroprotective effect of PS-519 may be due to the reduction of the secondary brain damage induced by the inflammatory reaction after the ischemic insult.

We have previously demonstrated that treatment with rhtPA at 4 hours after ischemia significantly induces upregulation of P-selectin, E-selectin, and ICAM-1 and exacerbates ischemic cell damage, suggesting that the inflammatory reaction may limit the efficacy of thrombolytic therapy. In the present study administration of PS-519 in conjunction with rhtPA at 2, 4, and 6 hours after ischemia significantly reduced ischemic infarct volume and myeloperoxidase-immunoreactive cells, indicating that PS-519 effectively reduces inflammation reaction after thrombolysis and consequently enhances the effectiveness of thrombolytic therapy.

The high risk of hemorrhagic transformation is an important limitation of thrombolytic therapy. However, recent studies show that only hemorrhage >30% of the infarct area with considerable space-occupying effect significantly increases the risk of clinical deterioration. Other types of hemorrhagic transformation are not associated with clinical deterioration. Treatment with rhtPA, although associated with a higher increase of hemorrhage, decreases the overall risk of disability and death at 3 months. In the present study administration of rhtPA alone at 4 hours after embolization significantly increased the total area of microscopic hemorrhage without reduction of cerebral infarction. Combination treatment with PS-519 and rhtPA at 2, 4, and 6 hours after embolization provided therapeutic benefit without increasing the incidence of hemorrhagic transformation, and a trend toward a reduction of hemorrhagic transformation was detected in all PS-519–treated groups compared with nontreated rats.

In summary, our data show that PS-519 provides potent neuroprotection at 2 and 4 hours after embolic focal ischemia. Combination treatment with PS-519 and rhtPA extends the neuroprotective effect to at least 6 hours after embolization without an increase in hemorrhagic transformation.
Acknowledgments

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