Use of a Poly(ADP-Ribose) Polymerase Inhibitor to Suppress Inflammation and Neuronal Death After Cerebral Ischemia-Reperfusion

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Background and Purpose—Most stroke patients do not present for medical treatment until several hours after onset of brain ischemia. Consequently, neuroprotective strategies are required with comparably long therapeutic windows. Poly(ADP-ribose) polymerase inhibitors such as PJ34 are known to suppress microglial activation, a postischemic event that may contribute to neuronal death. We evaluated the effects of PJ34 administered 8 hours after transient forebrain ischemia.

Methods—Rats were subjected to 10 minutes of forebrain ischemia and treated with PJ34 for 7 days beginning 8 hours after reperfusion. Activated microglia and infiltrating macrophages were evaluated at serial time points between zero and 14 days after ischemia by immunostaining for CD11b. CA1 neuronal survival was evaluated 7 days after ischemia.

Results—Rats treated with PJ34 showed a near-complete inhibition of microglia/macrophage activation (evaluated on day 5) and an 84% reduction in CA1 neuronal death.

Conclusions—Administration of PJ34 as late as 8 hours after transient ischemia–reperfusion has a large protective effect on CA1 survival. This effect may be mediated by suppression of the postischemic brain inflammatory response. (Stroke. 2007;38[part 2]:632-636.)

Key Words: inflammation ■ ischemia ■ microglia

Several pharmacological interventions have been shown to reduce brain injury in animal models of stroke when administered at short time points, generally zero to 3 hours, after onset of ischemia. These include glutamate receptor antagonists, free radical scavengers, protease inhibitors, and the thrombolytic agent, tissue-plasminogen activator (tPA).1–4 However, clinically useful agents must be efficacious when given at considerably longer intervals after onset of ischemia, because the vast majority of stroke patients do not present for medical treatment until substantially after this short time window. Such agents must target processes that contribute to cell death at later time points. One such process is the brain inflammatory response.

A prominent aspect of the brain inflammatory response is activation of microglial cells. Microglia are the resident macrophages of the central nervous system. They are derived from bone marrow precursors and have a slow interchange with the circulating macrophage pool.5,6 Microglia in normal brain have a highly branched "ramified" morphology that can rapidly transform into an activated, amoeboid morphology in response to stressors such as ischemia.7,8 Microglial activation precedes ischemic neuronal death,7,9,10 and the location of microglial activation correlates with neuronal death.7,11–13 Activated microglia can promote neuronal death by releasing glutamate, the NMDA receptor modulator d-serine, cytokines, reactive oxygen species, and proteases.8,14–20 At later time points, infiltrating bloodborne leukocytes also contribute to the brain inflammatory response.21,22

Many aspects of the inflammatory response are regulated by the transcription factor NF-κB. NF-κB requires, as a coactivating factor, the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1). Prior studies have shown a requirement for PARP-1 in NF-κB-mediated microglial gene transcription, integrin expression, morphologic changes, migration, and neurotoxicity.20,23–26 PARP-1 is similarly involved in NF-κB-mediated systemic inflammatory responses.27 These findings suggest that PARP inhibitors could be used to prevent inflammation in brain and thereby reduce ischemic brain injury. Because microglia require several hours to become fully activated, and even longer times are required for leukocyte infiltration from the bloodstream,21 this approach could have a longer therapeutic window of opportunity than many other neuroprotective strategies. In the present study, we examined this possibility using the PARP inhibitor N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide (PJ34) in a rat model of forebrain ischemia–reperfusion.
Methods

Materials

PJ34 was obtained from Inotek. Sprague-Dawley rats were obtained from Simonsen Laboratories and were housed two per cage under conditions of constant temperature (18°C), light from 6:00 AM to 6:00 PM, and free access to food and water.

Ischemia–Reperfusion

Transient forebrain ischemia was induced by the method of Smith et al.28 under a protocol approved by the San Francisco Veterans Affairs Medical Center animal studies committee. Male adult Sprague-Dawley rats weighing 250 to 300 g were anesthetized with 2% to 3% isoflurane in 70% NO2/balanced O2 through a nose cone. Once anesthetized, the animals were maintained on a small animal ventilator/anesthesia machine and the isoflurane was reduced to 1% to 2%. A femoral artery catheter was placed for withdrawal of blood and monitoring of blood pressure. Body temperature was maintained at 37±1.0°C by means of a heating blanket and heating lamp controlled by a rectal thermistor. Both common carotid arteries were exposed with a neck incision. The exposed carotid arteries were clamped and systemic mean arterial pressure was lowered to 40±5 mm Hg by withdrawing blood (7 to 10 mL) from the femoral artery into a heparinized syringe maintained at 37°C. Perfusion was restored 10 minutes later by unclamping the carotid arteries and reintroducing the blood to the femoral artery. The incisions were closed, the femoral artery catheter was removed, and anesthesia was discontinued. Rats assigned to the sham ischemia group underwent the same surgical procedure but with no blood withdrawal and no clamping of the carotid arteries.

Rats treated with PJ34 received an initial dose of 15 mg/kg by intraperitoneal injection in a volume of 1.0 mL of 0.9% saline at 8 hours postischemia and subsequent 15-mg/kg doses at 12-hour intervals until they were killed. Control groups received injections of 1 mL saline vehicle alone with the same dosing schedule.

Evaluation of Microglial Activation

Rats were killed at several time points after ischemia–reperfusion: 3, 6, 12, and 24 hours and 2, 3, 5, 7, and 14 days with n=2 to 3 at each time point. Brains were fixed by 4% paraformaldehyde perfusion and postfixed by immersion in 4% paraformaldehyde followed by 20% sucrose. Serial 50-μm cryostat sections were collected, and immunostaining was performed with a mouse antibody to rat CD11b (Serotec) at a 1:200 dilution as described previously.29 After washing, the sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) at a dilution of 1:500 for 2 hours at room temperature. Sections were then subsequently washed, mounted, and imaged with a Leica confocal laser scanning microscope. Secondary antibody alone without prior incubation with primary antibody showed no staining.

Evaluation of Neuronal Death

Rats were killed 7 days postischemia, and the brains were rapidly removed and flash frozen on powdered dry ice. Coronal 20-μm frozen sections were prepared, fixed in 70% ethanol, and stained with hematoxylin/eosin as described previously.30 To quantify neuronal death, we analyzed five coronal sections from each animal, beginning 4.0 mm posterior to bregma and spaced 200 μm apart. An observer blind to the treatment condition counted the number of eosinophilic cells in the hippocampal CA1 cell field in both hemispheres of each rat. A 10X objective microscopic field was centered on the structure of interest and the number of eosinophilic neurons in the field was recorded for each section. Data from each animal were expressed as the mean number of dead (eosinophilic) neurons per section. The group mean from the PJ34-treated rats (n=5) were compared with the saline-treated control group (n=5) with Student t test. Two sham surgery rats were also evaluated, and these showed no detectable neuronal death. The five rats assigned to the saline-treated control group exclude two rats that developed seizures and were killed within the first 48 hours after ischemia. None of the rats treated with PJ34 developed seizures.

Results

Preliminary studies were performed to confirm the effect of the PARP inhibitor on microglial activation in vivo. Activated microglia were evaluated by immunostaining for the surface marker CD11b, which permits assessment of both microglial morphology and CD11b expression level.31 Basal CD11b expression is punctate and disperse, reflecting the highly ramified morphology of resting microglia (Figure 1A).
After ischemia–reperfusion, the microglia of the CA-1 region upregulate CD11b expression, retract their processes, and take on an “amoeboid” morphology (Figure 1B through 1D). After 24 hours, infiltrating macrophages also contribute to this CD11b signal.21,22 This inflammatory response peaked at 48 to 72 hours and began to resolve after 7 days (not shown), a pattern very similar to that previously reported with ischemia model.7 Rats given PJ34 beginning 8 hours after the ischemic insult exhibited a near-complete suppression of this microglial response when evaluated at the 5-day time point (Figure 1). Other time points were not evaluated with this exact dosing regimen, but a similar, near-complete suppression of microglial activation at the 3-day and 7-day time points was observed with PJ34 administered beginning either 12 hours or 24 hours after ischemia–reperfusion (not shown).

We then assessed the effect of PJ34 on neuronal survival by standard hematoxylin and eosin histology at day 7 after ischemia–reperfusion (Figure 2). Rats receiving PJ34 beginning 8 hours after ischemia and treated every 12 hours for 7 days showed an 84% reduction in CA1 neuronal death relative to rats treated with saline vehicle alone. Rats treated with sham surgery showed no neuronal death.

Discussion
These studies provide proof-of-principle that administration of a PARP inhibitor at delayed time points after transient ischemia–reperfusion can prevent neuronal death. This reduction in cell death was associated with a near-complete suppression of the cellular brain inflammatory response.

The CD11b antigen is expressed not only by microglia, but also by activated bloodborne macrophages.31 Because macrophages infiltrate brain after ischemia, the reduction in CD11b immunoreactivity may reflect reduced macrophage infiltration in addition to reduced microglial activation.21,22,32 PJ34, and other PARP-1 inhibitors, can suppress the cellular inflammatory response in a variety of settings.33–35 and evidence suggests that this stems from the role of PARP-1 as a necessary cofactor for the transcription factor NF-κB.20,23–26 PJ34 is one of the most potent inhibitors of PARP-1 developed,36 and prior studies show that it readily crosses the blood–brain barrier.37–39 Although characterized as a PARP-1 inhibitor, it is likely that this agent also acts on other PARP family members. PJ34 does not have any known effects at other sites, but such effects cannot be excluded.

The efficacy of PJ34 administered as late as 8 hours after ischemia–reperfusion, coupled with the profound inhibition of microglial activation with this dosing strategy, supports the idea that the PJ34 neuroprotective effect is mediated by inhibition of microglial activation. A key and unique feature of the present study is repeated dosing with PJ34 to maintain suppression of the microglial response through the normal peak period of this response. We cannot entirely exclude the possibility that PJ34 might also act by a direct neuroprotective action on CA1 neurons. PJ34 is a potent inhibitor of the PARP-1 cell death pathway40,41; however, prior studies suggest that neuronal PARP-1 is activated only transiently after reperfusion,42 and neither PJ34 nor any other PARP inhibitor has been reported to prevent neuronal death when administered as long as 8 hours after ischemia. This point will be settled in future studies in which PJ34 dosing will be designed to target specifically only the cytotoxic or inflammatory aspects of PARP-1 activation.

The present studies were done in male rats only. Several studies suggest significant differences between male and females responses to PARP inhibitors, including responses to the antiinflammatory effects of PARP inhibitors.35,39 Thus, it will be important to extend these studies to include female subjects. Similarly, the 10-minute ischemia insult used for the study here most closely mimics the condition of cardiac arrest and resuscitation, and it will also be important to evaluate the effect of repeated PJ34 administration in a permanent focal ischemia model, which more closely approximates the more common clinical stroke scenario of cerebral artery occlusion. Finally, it should be noted that PARP inhibitors could also have deleterious effects in the setting of brain ischemia. PARP-1 is activated by DNA damage and plays a role in the repair of single strand DNA breaks. It follows that blocking this activity may lead to consequences associated with DNA mutations, namely apoptosis or oncogenesis, and there has been one report citing increased neuronal death in transient ischemia treated with a PARP inhibitor.42 Future studies will require observations at extended time points to fairly evaluate the long-term consequences of PARP inhibitors, particularly in settings in which they are given in repeated doses.

Brain inflammation has been recognized as a potential target for stroke treatment for several years, and various ap-
proaches have been tried to suppress aspects of postischemic brain inflammation. These approaches include hypothermia, inhibition of matrix metalloproteinases, antibody-mediated inhibition of neutrophil infiltration, lipopolysaccharide preconditioning, and the use of minocycline. Minocycline has recently been shown to be a very potent PARP inhibitor, and this may be the mechanism by which minocycline suppresses microglia activation. Results of the present study show that it is possible to prevent the death of hippocampal CA1 neurons after transient ischemia by administering the PARP inhibitor PJ34 as late as 8 hours after ischemia. We propose that this effect is attributable to the suppression of microglial activation, but the possibility of a direct neuroprotective effect cannot be entirely excluded on the basis of the studies completed. Future studies will address this point and also examine outcome after much longer survival times and in rats of both sexes.

Acknowledgments
Aaron Hamby designed the experiment, assisted in surgery, delivered all postoperative care and drug administration, performed histology and immunohistochemistry, performed cell counts, and assisted in manuscript preparation. Sang Won Suh performed all animal surgeries and assisted in cell counts. Tiina Kauppinen assisted in the experimental design, performed immunohistochemistry, and assisted in manuscript preparation. Raymond Swanson supervised the study and prepared the manuscript.

Sources of Funding
This work was supported by grants to R.A.S. from the Department of Defense and the National Institutes of Health, and by the Department of Veterans Affairs.

Disclosure
None.

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Stroke. 2007;38:632-636
doi: 10.1161/01.STR.0000250742.61241.79
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/2/632

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