Interaction Between Inducible Nitric Oxide Synthase and Poly(ADP-ribose) Polymerase in Focal Ischemic Brain Injury

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Background and Purpose—Overactivation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) contributes to ischemic brain injury. Because PARP upregulates proinflammatory genes, we investigated whether inducible nitric oxide synthase (iNOS), a gene involved in the deleterious effects of postischemic inflammation, participates in the mechanisms by which PARP activation contributes to cerebral ischemic injury.

Methods—The middle cerebral artery (MCA) was occluded in mice for 20 minutes using an intravascular filament, and injury volume was measured 72 hours later in Nissl-stained brain sections. mRNA expression was assessed in the postischemic brain by the quantitative “real-time” polymerase chain reaction.

Results—The PARP inhibitor PJ34 reduced infarct volume and attenuated postischemic iNOS mRNA upregulation by 72%. To determine whether iNOS contributes to the toxicity of PARP, the iNOS inhibitor aminoguanidine was co-administered with PARP inhibitors. Unexpectedly, co-administration of PARP and iNOS inhibitors, or treatment of iNOS-null mice with PARP inhibitors, abrogated the protective effect afforded by iNOS or PARP inhibition alone. The loss of neuroprotection was associated with upregulation of the inflammatory genes iNOS, intercellular adhesion molecule-1, and gp91phox.

Conclusions—The results suggest that iNOS expression contributes to the deleterious effects exerted by PARP activation in cerebral ischemia. However, iNOS activity is required for the protective effect of PARP inhibition and, conversely, PARP activity must be present for iNOS inhibition to be effective. The findings unveil a previously unrecognized deleterious interaction between iNOS and PARP that is relevant to the development of combination therapies for ischemic stroke. (Stroke. 2004;35:2896-2901.)

Key Words: adhesion molecules ■ inflammation ■ neuroprotection ■ polymerase chain reaction, real time

There is substantial evidence that poly(ADP-ribose) polymerase (PARP), a nuclear protein that participates in DNA base excision repair in response to DNA damage, is involved in the mechanisms of ischemic neuronal death.1 Thus, pharmacological inhibitors of PARP attenuate the brain injury produced by occlusion of the middle cerebral artery (MCA) in rodents, whereas mice lacking PARP1 are markedly protected from cerebral ischemic damage.2–4 Because PARP activation is a downstream pathogenic factor in a wide variety of cell injury models, it has been suggested that PARP activation represents a “final common pathway” for cell death.1

The mechanisms of the cytotoxicity linked to PARP activation have not been fully elucidated. One hypothesis is that activated PARP consumes NAD⁺, resulting in ATP depletion that worsens the energy deficit of the ischemic brain.5 Another hypothesis is that active PARP induces the release of the apoptosis inducing factor from mitochondria, which initiates caspase-independent cell death.6 A third possibility is that PARP acts as a co-activator of nuclear factor κB (NF-κB), a transcription factor that contributes to cell injury by upregulating the expression of potentially deleterious proinflammatory genes.7 Therefore, PARP-induced activation of NF-κB–dependent gene expression could play a role in the neurotoxicity resulting from PARP activation in the postischemic brain.

The “inducible” or “immunological” isoform of nitric oxide synthase (iNOS) is one of the NF-κB–dependent genes that contributes to ischemic brain injury.8 After focal cerebral ischemia, iNOS is expressed de novo in inflammatory and vascular cells, and its inhibition attenuates ischemic brain injury.9 Furthermore, mice lacking iNOS are relatively pro-
ected from the deleterious effects of focal cerebral ischemia. Therefore, iNOS could be one of the proinflammatory genes whose downregulation contributes to the neuroprotection afforded by PARP inhibitors.

In this study, we tested the hypothesis that iNOS contributes to the deleterious effect of PARP activation. In particular, we used a mouse model of transient focal cerebral ischemia to determine whether the protective effect of PARP inhibitors was still observed in the presence of iNOS inhibition or in iNOS-null mice. Our results support the hypothesis that iNOS contributes to the deleterious effect of PARP activation in the ischemic brain. Furthermore, they unveil a previously unrecognized and pathogenically relevant interaction between iNOS and PARP.

Materials and Methods

Mice
Experiments were performed in male C57BL/6 mice (age 2 to 3 months; weight 20 to 23 g; Charles River, Wilmington, Mass), and male iNOS-null mice (>10 backcrosses into C57BL/6) obtained from an in-house colony.

Transient MCA Occlusion
All procedures were approved by the Institutional Animal Care and Use Committee. Mice were anesthetized with a mixture of isoflurane (1.5% to 2%), oxygen, and nitrogen. A fiber optic probe was glued to the parietal bone (2 mm anterior and 5 mm lateral to bregma) and connected to a laser Doppler flowmeter (Periflux System 5010) for continuous monitoring of cerebral blood flow (CBF). For MCA occlusion, a heat-blunted black monofilament surgical suture 6-0 was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the MCA. The filament was left in place for 20 minutes and then withdrawn. Only animals that exhibited a reduction in CBF >85% during MCA occlusion and in which CBF recovered by >80% after 10 minutes of reperfusion were included in the study. This procedure leads to reliable infarcts involving both the cerebral cortex and the striatum. Laser Doppler flowmetry is not quantitative, but provides a reliable estimate of relative CBF. Rectal temperature was kept at 37.0±0.5°C during surgery and in the recovery period until animals regained consciousness.

Drug Treatments
Mice were randomly assigned to vehicle or treatment groups. The PARP inhibitor PJ34 (N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide.HCl; Inotek, Beverly, Mass; 10 mg/kg in saline, intraperitoneal) and administered 1 hour before and 2 hours after MCA occlusion. Another PARP inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1-(2H)-isouquinolinone (10 mg/kg in DMSO, intraperitoneal; DPQ; Sigma, St. Louis, Mo) was administered 2 hours before and 2 hours after MCA occlusion. The dose and administration protocol for PJ34 and DPQ have been shown to be effective in stroke models. The inhibitor of neuronal nitric oxide synthase (nNOS) 7-nitroindazole (25 mg/kg in DMSO, intraperitoneal; 7-Ni; Cayman Chemical, Ann Arbor, Mich) was given 1 hour before MCA occlusion. The iNOS inhibitor aminoguanidine (100 mg/kg in saline, intraperitoneal; AG; Sigma) was administered twice per day for 3 days starting 10 minutes after reperfusion. AG was administered earlier than in previous studies to assure that iNOS was inhibited at the time of PARP activation.

Infarct Volume Measurement
Brains were removed, frozen, and sectioned (thickness, 30 μm/L) in a cryostat. Brain sections were collected serially at 600-μm intervals and stained with cresyl violet. Infarct volume was determined using an image analyzer (MCID; Imaging Research Inc). To eliminate the contribution of postischemic edema to the volume of injury, infarct volumes were corrected for swelling as described.

Quantitative “Real-Time” Polymerase Chain Reaction
Experimental animals and sham-operated controls (n=4/group) were euthanized 6 hours, 24 hours, and 72 hours after reperfusion. Total RNA was prepared from the ischemic and contralateral hemispheres using Trizol reagent (Invitrogen Life Technologies, Carlsbad, Calif). Quantitative determination of gene expression levels, using a 2-step cycling protocol, was performed on Chromo 4 detector (Peltier Thermal Cycler MJ Research). Primers for iNOS (forward, 5'-CAGCTGGCTGTCACACACCT-3'; reverse, 5'-CATTGGAAGTCAAAGCTTTCCG-3'), ICAM (forward, 5'-GCTTGTGAGG-TGACTGAG-3'; reverse, 5'-GACGGAGCTGAAAGTGTGA-3'), and gp91(phox) (forward, 5'-GAGTTTTGCAGAAAGTCACTTCA-3'; reverse, 5'-GAGAGTTTCAGCCAAGGCTTC-3') were purchased from Invitrogen Life Technologies. Two microliters of diluted cDNA (1:10) was amplified by Platinum SYBR green qPCR supermix UDG (Invitrogen Life Technologies). The reactions were incubated at 50°C for 2 minutes and then 95°C for 10 minutes. A polymerase chain reaction cycling protocol consisting 15 seconds at 95°C and 1 minute at 60°C for 45 cycles were required for quantification. Relative expression levels were calculated by 2ΔΔ CT method. Quantities of all targets in test samples were normalized to the mouse HPRT housekeeping gene and values were normalized to vehicle-treated control hemispheres.

Statistical Analysis
Data are expressed as mean±SEM. Two-group comparisons were statistically evaluated by the Student t test. Multiple comparisons were evaluated by the analysis of variance and Fisher’s protected least significance difference (PLSD) test. Differences were considered significant at P<0.05.

Results
PARP Inhibitor PJ34 Attenuates Postischemic iNOS Expression
In vehicle-treated mice, iNOS mRNA was elevated at 24 and 72 hours after ischemia (Figure 1A). Treatment with PJ34 attenuated iNOS expression markedly (−72% at 72 hours; P<0.05; t test). To determine whether other proinflammatory genes also were downregulated, we studied the effect of PJ34 on ICAM-1 and gp91(phox) mRNA expression. PJ34 enhanced ICAM-1 mRNA at 6 hours, and attenuated it at 24 and 72 hours (−45% at 72 hours; P<0.05) (Figure 1B). In contrast, gp91(phox) mRNA was not attenuated by treatment with PJ34 at 24 and 72 hours, but it was enhanced at 6 hours (Figure 1C).

iNOS Inhibitor Aminoguanidine Abrogates the Protective Effect of PARP Inhibitors
To examine whether the attenuation of iNOS expression contributed to the neuroprotection afforded by PARP inhibitors, we studied the effect of co-administration of PARP inhibitors (PJ34 or DPQ) and the iNOS inhibitor AG. We reasoned that if the attenuation of iNOS expression contributes to the beneficial effect of PARP inhibitors, then cotreatment with AG and PARP inhibitors should not confer a protection greater than that provided by either agent alone. However, if the mechanism of the protection exerted by PARP inhibitors is not related to iNOS, cotreatment should confer additive protection. Administration of PJ34 or AG reduced ischemic injury by 49% and 53%, respectively.
However, cotreatment of AG and PJ34 abrogated the protective effect (Figure 2A). Similarly, the reduction in injury volume produced by the PARP inhibitor DPQ (−49%) was lost when DPQ and AG were administered together (Figure 2B). Treatment with PJ34, DPQ, and/or AG did not alter the CBF changes produced by ischemia and reperfusion (Figure 2C).

**PARP Inhibitors Do Not Reduce Injury Volume in iNOS-Null Mice**

We then tested the effect of PARP inhibitors in iNOS-null mice. Consistent with previous reports,10 iNOS-null mice had smaller infarcts than wild-type mice (−44%; P<0.05). However, as with the iNOS inhibitor AG, administration of DPQ or PJ34 abolished the protection observed in iNOS-null mice (Figure 3).
7-NI Does Not Abolish the Protective Effect of PJ34
To determine whether inhibition of other NOS isoforms also abrogated the protection exerted by PARP inhibition, we tested the effect of co-administration of PJ34 and the nNOS inhibitor 7-NI. 7-NI reduced ischemic injury (−52%; P < 0.05 from vehicle), but at variance with AG, co-administration of PJ34 and 7-NI did not increase injury volume (Figure 4; P > 0.05 from 7-NI).

Cotreatment With PJ34 and AG Increases iNOS, ICAM-1, and gp91phox mRNA Expression 72 Hours After Ischemia
We then examined the effect of cotreatment with PJ34 and AG on the expression of iNOS, ICAM-1, and gp91phox. As illustrated in Figure 5, PJ34 and AG, when administered separately, attenuated the expression of iNOS and ICAM-1 mRNA. However, co-administration of these agents upregulated the expression of iNOS and ICAM-1, as well as gp91phox (Figure 5).

Discussion
We tested the hypothesis that iNOS contributes to the deleterious effect of PARP activation in focal cerebral ischemia. We found that the PARP inhibitor PJ34 attenuates iNOS mRNA expression in the postischemic brain, an effect associated with a reduction in ischemic injury. However, the protective effect afforded by the PARP inhibitors PJ34 or
DPQ was lost when either of these agents was co-administered with the iNOS inhibitor AG. Furthermore, treatment of iNOS-null mice with PJ34 or DPQ enlarged the infarct. To provide insight into the molecular changes underlying these effects we investigated the expression of inflammation-related genes iNOS, ICAM-1, and the essential NADPH oxidase subunit gp91phox. We found that cotreatment with iNOS and PARP inhibitors enhances the expression of these potentially deleterious genes. The findings indicate that the protective effect exerted by PARP inhibitors requires the presence of iNOS for its full expression and, vice versa, the presence of PARP activity is needed for the protection exerted by iNOS inhibition. These observations provide evidence for a previously unrecognized interaction between PARP and iNOS in the setting of focal cerebral ischemia.

The findings of the present study cannot be attributed to nonspecific pharmacological effects of the inhibitors used. As for PARP inhibitors, the findings with PJ34 were replicated by the mechanistically unrelated PARP inhibitor DPQ.1 As for iNOS, the findings obtained with AG were identical to those obtained in iNOS-null mice. Therefore, the exacerbation of injury by PARP inhibitors + AG is not related to nonspecific actions of AG, or to potential deleterious effects of early administration of this inhibitor.15 Furthermore, the findings cannot be attributed to hemodynamic effects of the inhibitors that altered the intensity of the ischemic insult, because the neuroprotection afforded by iNOS and PARP inhibitors is not mediated by effects on CBF,2,9 and is not associated with changes in intra-ischemic and postischemic CBF. Finally, the lack of reduction in injury volume in iNOS-null mice is not caused by the fact that the stroke was maximally reduced, because 7-NI attenuates stroke volume in iNOS-null mice.13

Our finding that PJ34 attenuates iNOS expression suggests that iNOS-derived NO contributes to the deleterious effects of PARP activity in ischemia. The mechanisms by which PARP contributes to ischemic brain injury have not been fully elucidated. Although activated PARP could be deleterious by inducing ATP depletion and aggravating the energy deficit,1,5 this is unlikely to be the case in ischemia-reperfusion models because ATP levels are not substantially attenuated.16 It has also been suggested that PARP activity leads to cell death by translocation of the apoptosis-inducing factor from the mitochondria to the nucleus.6 However, the mechanisms of this effect have not been elucidated in full,17 and its relevance in vivo needs to be established. The findings of the present study provide evidence that reduction of the expression of iNOS and other inflammatory genes is another mechanism by which PARP inhibition could attenuate ischemic brain injury.

It is generally assumed that larger infarcts have a larger inflammatory component.18 Therefore, the reduction in iNOS expression produced by PJ34 could be caused by an attenuated inflammatory response related to the 50% reduction in infarct volume. However, the reduction in iNOS expression afforded by PJ34 (~72%) was much greater than that produced by AG (~45%), despite comparable reductions in lesion volume. Furthermore, AG or PJ34 attenuated the expression of ICAM equally, whereas the expression of gp91phox mRNA was not reduced, despite the smaller infarct. Therefore, the changes in mRNA expression induced by PJ34 cannot be explained entirely by a reduction in injury volume.

Several lines of evidence support the view that PARP activity promotes inflammation. First, PARP inhibition attenuates NF-κB gene-dependent transcription in a variety of cells, including microglia.7,19 Second, the expression of inflammation-related genes is attenuated in PARP1-null mice with intestinal ischemia-reperfusion injury.20 Third, PJ34 treatment attenuates iNOS and ICAM-1 expression in the spinal cord of mice with experimental allergic encephalomyelitis.21 iNOS and ICAM-1 are well known to contribute to ischemic brain injury.8 Therefore, the results of these studies, in concert with the present findings, provide evidence that one of the mechanisms by which PARP inhibition reduces ischemic injury is attenuation of postischemic inflammation. However, the observation that gp91phox, an essential subunit of the free radical-producing enzyme NADPH oxidase,22 was not reduced suggests that not all inflammation-related genes are attenuated by PARP inhibition.

Surprisingly, however, the protection exerted by PARP inhibitors was abolished by iNOS inhibition, an effect associated with an enhancement of expression of iNOS, ICAM-1, and gp91phox. Therefore, if both PARP and iNOS are inhibited, then postischemic inflammation is exacerbated and the resulting tissue damage is increased, leading to loss of the protective effect conferred by iNOS or PARP inhibition alone. The fact that iNOS, ICAM-1, and gp91phox mRNAs were upregulated in the presence of PARP and iNOS inhibitors suggests that PARP activity is not an absolute requirement for the expression of these proinflammatory genes in the postischemic brain. Deleterious effects resulting from iNOS, ICAM-1, and gp91phox overexpression8,23,24 are likely mechanisms for the worsening of the injury produced by iNOS + PARP inhibition. This hypothesis, however, needs to be tested in future studies. Furthermore, it remains to be determined whether these changes in mRNA expression are associated with the expected changes in protein expression, leading to the anticipated biological actions.

Unlike AG, the nNOS inhibitor 7-NI did not abrogate the protective effect of PARP inhibition. Furthermore, the protective effects of PJ34 and 7-NI were not additive, a finding consistent with the hypothesis that nNOS-derived NO leads to formation of peroxynitrite, which, in turn, leads to PARP activation by producing DNA damage.25 This conclusion is supported by experiments in which PARP activation was found to be reduced by treatment with 7-NI or in nNOS-null mice.26 In contrast, administration of the iNOS inhibitor AG in mice treated with PJ34 abrogated the protective effect conferred by these inhibitors when administered alone. Therefore, iNOS-derived NO interacts with active PARP in a way that is different from nNOS-derived NO.

Irrespective of the mechanisms of the reciprocal interactions between iNOS-derived NO and PARP, the present results suggest caution when evaluating combination therapies for stroke. There is an increasing interest in testing the effect of more than one agent on the outcome of cerebral ischemia.27 Combination therapy is based on the assumption
that neuroprotective agents, when administered together, provide a greater protection than that conferred by each agent alone. Although there is evidence supporting the validity of this approach, the choice of the agents to be co-administered has to be carefully weighted. The present results indicate that combination therapy can also produce deleterious consequences. Therefore, agents to be co-administered have to be carefully considered and tested exhaustively in a preclinical setting.

In conclusion, we have demonstrated that the protective effect of PARP inhibition in a model of focal cerebral ischemia is accompanied by attenuation of iNOS mRNA expression. However, co-administration of iNOS and PARP inhibitors worsened ischemic injury, unveiling an unforeseen deleterious effect of the combined inhibition. The worsening of the damage is associated with an increase in the expression of potentially deleterious genes such as ICAM-1 and gp91^phox. Although these findings suggest that iNOS participates in the mechanisms by which PARP activity contributes to ischemic injury, they also suggest a previously unrecognized and pathogenically relevant interaction between iNOS and PARP. That is, PARP activity is needed for the expression of the protective effect of iNOS inhibition, and iNOS-derived NO is required for the protection exerted by PARP inhibition. These interactions have to be taken into account when developing combination strategies for the treatment of cerebral ischemia in humans.

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