Poly(ADP-Ribose) Polymerase Impairs Early and Long-Term Experimental Stroke Recovery

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Background and Purpose—Poly(ADP-ribose) polymerase (PARP-1; Enzyme Commission 2.4.30) is a nuclear DNA repair enzyme that mediates early neuronal ischemic injury. Using novel 3-dimensional, fast spin-echo–based diffusion-weighted imaging, we compared acute (21 hours) and long-term (3 days) ischemic volume after middle cerebral artery (MCA) occlusion in PARP-1–null mutants (PARP−/−) versus genetically matched wild-type mice (WT mice). PARP−/− mice were also treated with viral transfection of wild-type PARP-1 to determine whether protection from MCA occlusion is lost with restoration of the gene product.

Methods—Halothane-anesthetized mice were treated with reversible MCA occlusion via intraluminal suture technique. Ischemic volumes were delineated by diffusion-weighted imaging with high spatial and temporal resolution during MCA occlusion and reperfusion. Recombinant Sindbis virus carrying β-galactosidase (lacZ) or PARP-1 was injected into ipsilateral striatum, then animals underwent MCA occlusion 3 days later. Infarction volume was measured at 22 hours of reperfusion (2,3,5-triphenyltetrazolium chloride histology).

Results—Reduction in regional water apparent diffusion coefficient (ADC) during occlusion or secondary ADC decline during reperfusion was not different between groups. Ischemic volume was smaller early in occlusion in PARP−/− versus WT mice and remained less at 21 hours of reperfusion. Ischemic volume then increased from 1 to 2 days in all mice, then stabilized without further change. Ischemic damage was smaller in PARP−/− than in WT mice at 3 days. Transfection of PARP-1 into PARP−/− mice increased stroke damage relative to lacZ-injected PARP−/− and increased damage to that of the WT mice. Intraischemic laser-Doppler flowmetry and physiological variables were not different among groups.

Conclusions—PARP-1 deficiency provides both early and prolonged protection from experimental focal stroke. The mechanism is not linked to preservation of ADC and mitigation of secondary energy depletion during early reperfusion. (Stroke. 2002;33:1101-1106.)

Key Words: adenosine diphosphate ribose ■ cerebral ischemia ■ magnetic resonance imaging ■ Sindbis virus ■ mice

Poly(ADP-ribose) polymerase (PARP-1; Enzyme Commission 2.4.30) is a nuclear enzyme activated by DNA strand nicks that occur after a variety of cell stressors. PARP-1 appears to be an important component in neuronal death pathways initiated by experimental stroke and in N-methyl-D-aspartate (NMDA), nitric oxide, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity.1–4 PARP-1 deficiency generated via knockout technology reduces early ischemic damage,1,2 although the precise mechanism(s) by which neuronal damage is averted remains unclear. On activation, the enzyme hydrolyzes nicotinamide adenine dinucleotide (NAD) and ribosylates a variety of nuclear proteins, thus consuming energy to place-mark and facilitate DNA repair.5 Therefore, one proposed mechanism for the detrimental role of PARP-1 in stroke is that the enzyme is excessively activated and consumes ATP in energy-depleted, peri-ischemic cells.1,2,6 We and others have shown that early infarction after middle cerebral artery (MCA) occlusion in PARP-1–null mutants (PARP−/−) is strikingly reduced relative to wild-type mice (WT mice).1,2 To further evaluate the importance of PARP in temporal evolution of ischemic damage, we used novel 3-dimensional, fast spin-echo diffusion MRI to quantify ischemic volumes during occlusion and over 3 days of recovery in PARP−/− mutants. In a second set of experiments, we evaluated the importance of PARP-1 deficiency per se in cerebral ischemia rather than developmental compensation or genetic influences within PARP−/− mice. We employed a viral transfec-

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tion method using recombinant replication-deficient Sindbis virus carrying either the reporter gene β-galactosidase (lacZ) or wild-type PARP-1. Sindbis virus containing wild-type PARP-1 demonstrates significant PARP-1 activity in vitro, while virus containing lacZ is devoid of PARP-1 catalytic activity.4 We tested the hypothesis that reintroduction of PARP-1 protein in PARP−/− brain restores vulnerability to ischemia and increases infarction volume after MCA occlusion.

Materials and Methods

Animals

The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Institutional Animal Care and Use Committee. Studies were conducted in male mice homozygous for PARP-1 deficiency (PARP−/− mice) and wild-type 129 Sv/Ev mice (WT mice). PARP−/− mice were bred on a pure 129 Sv/Ev background to avoid effects of differing genetic strains, as previously described.1,7 Our colony is maintained by outbreeding with purebred 129 Sv/Ev WT mice (Taconic, Germantown, NY) to minimize inbreeding effects and to ensure that PARP−/− mice are of the same strain as WT mice studied as control cohorts.

Ischemic Model

PARP−/− or WT mice (body weight, 20 to 32 g) were anesthetized with 1% to 1.2% halothane in oxygen-enriched air via nose cone, then treated with unilateral, reversible MCA occlusion, as previously described.1,8,9 In brief, a 5-0 nylon monofilament is inserted via an external carotid artery stump to a point 6 mm distal to internal carotid artery/pterygopalatine bifurcation, and the common carotid artery is temporarily occluded with a 6-0 silk suture. Ischemia is verified at 30 minutes of occlusion by awakening the mouse and testing for neurological deficits and abnormal motor function, as previously described. To establish reperfusion, the monofilament is withdrawn, and the carotid suture is removed.

Imaging Experiments

A novel 3-dimensional, diffusion-weighted imaging (DWI) technique, with the use of a fast spin-echo sequence with modifications as recently described,10 was applied to study MCA occlusion and early recovery. The technique allows sequential whole-brain images with high spatial (0.17×0.25×0.5 mm) and temporal (7 minutes) resolution. All studies were performed on a 4.7-T GE Omega nuclear MR system with triple gradient axes. Mice were positioned within the MRI cradle equipped with a stereotaxic head holder and 2-cm-diameter surface coil. The data matrix was 64×32×16 and was zero-filled in all 3 directions to 128×64×32, with a field of view of 22×16×16 mm. The resolution in the slice direction was 0.5 mm per slice after zero-filling. Because of relatively short T2 values (approximately 60 ms) at the field of 4.7 T, the echo train was limited to 8 echoes, during which the signal decays to approximately 40%. The maximal DW images were recorded with a diffusion gradient duration δ of 5 ms and a gradient strength G1 of 12 G/cm along all 3 directions. The diffusion time Δ was 17.5 ms. The effective echo time was 30 ms, and the duration between successive echoes was 5.7 ms. With a repetition time of 1 second, the scanning time was 64 seconds per image. For diffusion weighting, 4-shot tetrahedral isotropic weighting was used (b=1224 s/mm²). An additional least DW image was also acquired with b=70 s/mm² for water apparent diffusion coefficient (ADC) mapping. Thus, a total of 5 images were used for each trace ADC map (7 minutes per ADC map, including time required for data storage).

Animals were randomized into 2 protocols: 60 minutes of reversible MCA occlusion with serial MRI for 21 hours of reperfusion or 60 minutes of occlusion with daily MRI for 3 days of recovery. In the acute recovery experiments, mice were immediately placed in the magnet after occlusion, and 2 sets of DW images were recorded at 10 minutes and 50 minutes. At 60 minutes, mice were removed from the magnet and the filament was withdrawn, allowing reperfusion. DW images were acquired every 10 minutes for the initial 50 minutes of reperfusion. Mice were further imaged for both ADC and T2-weighted images at 6 and 21 hours of reperfusion. Brains were then perfusion-fixed in 10% buffered formalin and cryoprotected with 30% sucrose in 0.1 mol/L sodium phosphate buffer (pH 7.4) for cresyl violet histology and quantification of infarction volume (Inquiry 3, Louts Inc) in 12 evenly spaced, mounded horizontal sections. Horizontal sections were used only for this experiment to better correlate tissue histology with MR image planes. In the long-term survival protocol (n=12 per group), early reperfusion DW images were omitted, and both DW T2-weighted images were acquired once a day for 3 days.

Viral Transfection

Replication-deficient mouse Sindbis virus (pSR5) was used for expression of lacZ or PARP-1 constructs, as previously described.4 Sindbis is a mouse neurotrophic virus that predominately infects neurons by non–receptor-mediated internalization.11,12 This expression vector was chosen for its small genome size (approximately 10 kb), which allows substantial packaging of large DNA inserts, its ease of construction, and a high transfection efficiency.12–14 In brief, lacZ or wild-type PARP-1 was slubbed into the pSINRep5 plasmid (Invitrogen), linearized, then transcribed in vitro. RNA was transfected into baby hamster kidney (BHK) cells by electroporation, then the cells were trypsinized, washed, and resuspended in RNase-free PBS. RNA (10 μg) was added, and the cells were electroporated, plated in complete minimal essential medium, and incubated for 24 to 72 hours. The medium was used to transfect BHK cells that were then harvested at 3 days and pelleted. The pellet was freeze-thawed repeatedly in 10 mmol/L Tris with MgCl₂ and centrifuged, and the combined supernatants were filtered. The virus was amplified, then purified over a sucrose gradient. After large-scale purification,4 the medium was removed, and the virus band was extracted from the 20%/55% interface. Aliquots were stored at −80°C until thawed for intrastrial injection.

Halothane-anesthetized PARP−/− and WT mice were positioned in a head frame and injected with 3 μL of viral suspension (10⁶ plaque-forming units per milliliter) directly into striatum (from bregma 0.5 mm rostral, 2 mm lateral, 3.4 mm ventral; 20-minute infusion time). The suspension was injected through a silica tube fixed to the frame, with the use of a Hamilton pump syringe system (infusion syringe size 50 μL, 1.031 mm in diameter). The tube was withdrawn, the burr hole (0.3 mm in diameter) was sealed with sterile bone wax, and the skin wound was closed with 6-0 suture. Whole brain was harvested for X-gal staining and observation of β-galactosidase activity at 3, 4, and 5 days after injection (n=3 per time point). The tissue was perfusion-fixed in 4% formaldehyde, then postfixed for 2 to 4 hours at 4°C. Brain was sectioned (40 μm thick) on freezing microtome, then mounted and air dried overnight. Slides were incubated in PBS with 1 mm MgCl₂, then soaked overnight in X-gal solution (5-bromo-4-chloro-3-indolyl-β-d-galactoside) at 37°C.

PARP−/− and WT mice (weight, 21 to 27 g) were injected with viral constructs carrying either lacZ or wild-type PARP-1 (n=10 per group), then underwent MCA occlusion (2 hours) at 3 days after injection. Brains were harvested at 22 hours of reperfusion for quantification of infarction volume in standard coronal sections by cresyl violet histology and quantification of infarction volume in standard coronal sections by 2,3,5-triphenyltetrazolium chloride (TTC) staining and image analysis, as we described in our initial study of PARP−/− mice and other strains.1,15 In companion cohorts, arterial blood gases and blood pressure were determined at baseline, during occlusion, and at 30 minutes of reperfusion. Laser-Doppler flowmetry was used to confirm adequacy of occlusion and reperfusion in these animals.1,8,9 All data are expressed as mean±SE. Ischemic volumes as determined by MRI were analyzed by 1-way ANOVA and post hoc Newman-Keuls test to determine differences between groups at each time point. Histology was analyzed by 1-way ANOVA with post hoc Newman-Keuls test to detect differences between groups. The criterion for statistical significance was set at P<0.05. In the MRI
studies, animals with premature mortality (ie, those that did not survive to the end point of 21 hours or 3 days) or those in which there was a failure to produce full vascular occlusion were excluded. In the 21-hour study, 5 of 14 WT mice and 3 of 15 PARP/H11002 mice were excluded. In the 3-day study, 2 of 14 WT mice and 6 of 15 PARP/H11002 mice were excluded.

Results

MRI Studies
To define absolute change in averaged ADC over time in predefined, anatomic locations, 2 representative slices with clear visualization of ipsilateral and contralateral cortex and caudate putamen were studied. ADC in nonischemic brain was 0.65±0.03×10⁻³ cm²/s. During occlusion, ADC dropped in ischemic cortex and caudate (Figure 1). The amount of ADC reduction did not differ in PARP/H11002 versus WT mice, nor did recovery during reperfusion differ between groups. Figure 2 shows infarction volume, as determined from ADC images. For volume measurement, the entire low-ADC region was manually delineated in a slice-by-slice manner throughout whole brain, as previously described. An investigator blinded to animal treatment assignment traced boundaries of each region of interest per slice, detecting the edge where ADC abruptly changed. Infarction volume was smaller in PARP/H11002 than in WT mice during early occlusion. Injury volume decreased during immediate reperfusion in both groups but progressed over the remaining 21 hours, to a lesser extent in PARP/H11002 than in WT mice. Terminal histology (21 hours) in horizontal slices matched to the MRI slices indicated that damage was less in PARP/H11002 than in WT mice (37±7 [n=9] and 13±3 mm³ [n=9], respectively; P=0.007).

Infarction volume as determined by T2 imaging increased from 1 to 2 days of reperfusion in both WT and PARP/H11002 mice, then stabilized without further change at 3 days (Figure 3). Volume was smaller in PARP/H11002 mice (n=12) at 3 days after ischemia than in WT mice (n=9) (P=0.05). Similar results were obtained by analyzing ADC images (data not shown).

Transfection Studies
Intraparenchymal injection produced intense X-gal staining in striatum and small, scattered patches throughout the cortex ipsilateral to injection, indicating successful transfection of lacZ reporter gene to target MCA regions (Figure 4). Staining was present by 3 days after injection and persisted to 5 days. Infarction volume as percentage of contralateral hemisphere is summarized for lacZ-injected and PARP-1-transfected groups (Figure 5; n=10 per group). Striatal transfection of slice, detecting the edge where ADC abruptly changed. Infarction volume was smaller in PARP/H11002 than in WT mice during early occlusion. Injury volume decreased during immediate reperfusion in both groups but progressed over the remaining 21 hours, to a lesser extent in PARP/H11002 than in WT mice. Terminal histology (21 hours) in horizontal slices matched to the MRI slices indicated that damage was less in PARP/H11002 than in WT mice (37±7 [n=9] and 13±3 mm³ [n=9], respectively; P=0.007).

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PARP-1 into PARP−/− brain increased stroke damage relative to PARP−/− + lacZ in both cortex (transfected versus lacZ: 62±7% versus 39±7% contralateral cortex; P=0.03) and striatum (93±4% versus 69±10% contralateral striatum; P=0.03). Cortical infarction was smaller in lacZ-injected PARP−/− relative to WT-lacZ (PARP−/− versus WT: 39±7% versus 60±6% contralateral cortex; P=0.04), but striatal infarction was not different in this series of animals (69±10% versus 85±7% contralateral striatum; P=0.08). Intraischemic laser-Doppler flowmetry, blood pressure, blood gases, and body temperature were not different among experimental groups (Table).

Discussion
This report confirms our previous finding that PARP-1-deficient mice are strongly protected from experimental stroke1 and extends this observation with 4 important results. First, neuroprotection conferred by PARP-1 deficiency is not limited to the first 24 hours of reperfusion and can be demonstrated by MRI over 3 days in the maturing focal injury. Second, energy depletion, as measured by reduction in ADC to subnormal values, is not mitigated in PARP−/− mice. Furthermore, secondary energy failure is not ablated in PARP−/− mice because a delayed decay of regional ADC was observed in striatum over time to an amount similar to that in WT mice. Third, reduction in stroke volume in PARP−/− versus WT mice, as previously reported at 24 hours by standard histology,1,2 was observed by DWI at a very early stage of the insult. Fourth, restoration of wild-type PARP-1 protein in the mutant mouse effectively eliminates the protection ordinarily conferred by genetic deletion of PARP-1. These results suggest that PARP-1 is an important component of cell death pathways in brain, by a mechanism that is not necessarily linked to preservation of energy in the early peri-ischemic period.

PARP-1 is a nuclear zinc-finger DNA binding protein, constitutively expressed and catalytically activated by naked DNA. The enzyme is known as a molecular “nick sensor” and has been historically used as an experimental beacon for defective DNA in vitro and in vivo.16,17 Localization via in situ hybridization and immunocytochemistry shows that PARP mRNA and protein are expressed fairly ubiquitously in brain, with significant labeling over medium-size neurons throughout the central nervous system.18 Because transcriptional upregulation is not required, the enzyme is well suited as an early responder to protect vulnerable single-strand DNA disruptions. Accordingly, the activation of PARP is thought to precede that of other components of DNA repair such as DNA-dependent protein kinase activation and p53 induction.19 Via its N-terminal binding domain, PARP binds nuclear targets. This ADP ribosylation is accomplished by cleavage of NAD+ into ADP-ribose and nicotinamide and consumes 4 molecules of ATP to regenerate NAD+. Therefore, the activation of PARP is at high metabolic cost and is thought to rapidly deplete intracellular NAD+ concentration when excessively activated, slowing glycolytic rate, electron transport, and ATP generation and thus enhancing cell death. Alternatively, PARP inhibitors such as 3-aminobenzamide (3-AB) can block PARP excision repair activity and decrease neuronal survival after sublethal global cerebral ischemia.20 Others have proposed that PARP activation and its consequences for postis-

Figure 4. Photomicrograph of intense X-gal staining in mouse striatum and small, scattered patches throughout the ipsilateral hemisphere 5 days after injection of recombinant Sindbis virus lacZ reporter construct (SR5lacZ, 3 μL).

Figure 5. Infarction volumes at 22 hours in virus-injected mice, expressed as percentage of contralateral hemisphere. Tissue was evaluated in coronal sections by TTC staining. Groups were WT or PARP−/− mice injected with recombinant replication-deficient Sindbis virus carrying reporter gene β-galactosidase (+lacZ) or wild-type PARP-1 (+PARP-1) (n=10 per group). Mice were injected 3 days before MCA occlusion (2 hours). *P=0.05 vs WT lacZ-injected cohort; **P=0.05 vs PARP−/− lacZ-injected cohort.
chemic CA1 neuronal survival may hinge on NAD⁺ availability.

PARP-1 knockout mice have been demonstrated to be resistant to focal cerebral ischemia and oxidant stress in several experimental models. However, previous experiments using either transgenic animals or pharmacological inhibition have focused on acute protection without excluding the possibility that cell death is simply delayed rather than averted. We show here for the first time that PARP-conferred neuroprotection is still robust at 3 days after ischemia, suggesting that protection is long lasting. This observation agrees with the recent finding that PARP−/− mice are protected at 3 weeks from intrastriatal NMDA, but not α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), injection.

PARP-1 is activated within 2 hours of excitotoxic NMDA injection, within 1 hour, or 4 hours, or 24 hours of global forebrain ischemia, and within 48 hours after cardiac arrest. PARP inhibition (3-AB) has been shown to preserve cortical NAD⁺ in global cerebral ischemia over a narrow dose window but to have no effect on ATP depletion. NAD⁺ reduction at 2 and 24 hours after reversible MCA occlusion is mitigated in PARP−/− mice and in 3-AB–treated mice. However, the importance of partial NAD⁺ preservation to posts ischemic tissue redox state and energy metabolism is unclear because NADH/NAD⁺ ratios and concomitant ATP level have not been examined in previous studies. The present data suggest that a differential in severity of energy depletion over the first 21 hours of reperfusion does not readily explain amelioration of histological injury in PARP−/− versus WT mice. We observed no difference in the extent of the ADC drop during vascular occlusion, although there was clearly a difference in the volume of PARP−/− versus WT brain with low ADCs. Changes in ADC have been closely correlated to the time course of tissue energy metabolism during acute ischemia in nonmurine species and in mouse. Although the precise mechanism behind ADC depression in ischemia is not known, depletion of high-energy phosphates, acidosis, membrane depolarization, and intracellular sodium and water influx lead to a fall in regional ADC. Our observation that ADC initially recovered to that observed in nonischemic tissue, then fell at 21 hours of reperfusion, is consistent with previous reports of secondary energy failure. The time course and severity of ADC change were not altered in PARP−/− brain. On the other hand, stroke volumes, as defined by low ADC, were significantly smaller in PARP−/− mice from the first measurement during occlusion. This result could suggest that tissues in penumbral regions with low oxygen supply experienced less ATP depletion (or less consumption) and therefore maintained membrane potential. If so, this should be considered in the protective mechanism(s) at work in PARP−/− brain. As expected, cortical and striatal regions initially observed to be ischemic by DWI persisted in this state over days and progressed to infarction.

We questioned whether the protected phenotype of PARP−/− mice is explained by absence of gene product or is due to compensatory physiology in the unconditional knockout mouse. Recent experiments have revealed the existence of several PARP homologues in mammals (PARP-2, -3 and PHSP or Vault PARP). Several of these homologues have biological relevance to DNA repair processes and could be important to cerebral ischemia. To address the question of whether loss of PARP-1 gene product actually confers resistance to cerebral ischemia in PARP−/− mice, we reintroduced wild-type PARP-1 into these mice using a recombinant replication-deficient Sindbis virus, as previously described. Sindbis virus transfection has been successfully used in studies of neuronal proteins because of its short-term, high-yield gene product expression and preference for neuronal targets. The replication-deficient construct has been engineered to attenuate cytotoxicity. Accordingly, intrastrial injection in our model did not appear to cause inflammatory changes by gross pathological inspection at 3 or 5 days after transfection but only resulted in faint signs of injection trauma. However, virus containing wild-type PARP-1 significantly increased infarction volume in PARP−/− mice, eliminating the protection observed in PARP−/− relative to WT mice injected with Sindbis carrying lacZ. Furthermore, injection of virus containing lacZ in PARP−/− mice failed to increase infarct size as assessed at 22 hours after occlusion. These results indicate that ischemic vulnerability is a consequence of PARP-1 rather than a compensatory mechanism potentially inherent in the genetically engineered mice.

Several alternative hypotheses may better explain the importance of PARP-1 in ischemia/reperfusion than the "suicide theory," in which enzyme activation and excessive energy depletion lead to cell death. First, several important nuclear proteins are ADP-ribosylated once PARP-1 is activated, includ-

### Physiological Measurements

<table>
<thead>
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<th>Group</th>
<th>Weight, g</th>
<th>Time</th>
<th>pH</th>
<th>PaO₂, mm Hg</th>
<th>PaCO₂, mm Hg</th>
<th>Rectal Temperature, °C</th>
<th>MAP, mm Hg</th>
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<tr>
<td>WT + lacZ (n=6)</td>
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<td></td>
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<td>Ischemia</td>
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<tr>
<td>WT + PARP-1 (n=5)</td>
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<td>95±3</td>
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<td>PARP−/− + lacZ (n=2)</td>
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<td>36.7±0.0</td>
<td>97±7</td>
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MAP indicates mean arterial blood pressure; LDF, end-ischemic laser-Doppler flowmetry. Values are mean±SE.
ing topoisomerase, histones, DNA polymerase, and PARP itself \(^{5,6}\) (for review, see Reference 17). We speculate that alterations in these proteins could have a profound effect on cell repair and survival after intense cerebral ischemia. Second, PARP-1 inhibition results in attenuation of NMDA-induced glutamate efflux, suggesting that excessive PARP activation could amplify ischemic excitotoxicity in vivo.\(^{23}\) Third, PARP-1 has been strongly implicated in inflammatory processes such as endotoxic shock, likely acting through interactions with nuclear factor-\(\kappa B\) (NF-\(\kappa B\)).\(^{17,33-34}\) PARP-1 inhibitors block expression of proinflammatory mediators known to be integral to cerebral ischemic damage, including inducible nitric oxide synthase, interleukin-\(6\), and tumor necrosis factor-\(\alpha\).\(^{32-34}\) Recent evidence suggests a functional relationship between PARP-1 activation and NF-\(\kappa B\) induction in PARP-1-deficient cells.\(^{34}\) Therefore, it is possible that PARP-1 contributes via NF-\(\kappa B\) induction to production of inflammatory mediators during reperfusion.

In conclusion, these results emphasize that PARP-1 deficiency produces significant in vivo protection that persists well into the period of postischemic tissue injury maturation. PARP-1 activation represents a potentially key event in neuronal death pathways, as well as an avenue for therapeutic intervention.

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References


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