Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia

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Nitric oxide (NO) and peroxynitrite, formed from NO and superoxide anion, have been implicated as mediators of neuronal damage following focal ischemia, but their molecular targets have not been defined. One candidate pathway is DNA damage leading to activation of the nuclear enzyme, poly(ADP-ribose) polymerase (PARP), which catalyzes attachment of ADP ribose units from NAD to nuclear proteins following DNA damage. Excessive activation of PARP can deplete NAD and ATP, which is consumed in regeneration of NAD, leading to cell death by energy depletion. We show that genetic disruption of PARP provides profound protection against glutamate-NO-mediated ischemic insults in vitro and major decreases in infarct volume after reversible middle cerebral artery occlusion. These results provide compelling evidence for a primary involvement of PARP activation in neuronal damage following focal ischemia and suggest that therapies designed towards inhibiting PARP may provide benefit in the treatment of cerebrovascular disease.

Poly(ADP-ribose) polymerase (PARP, EC 2.4.4.30) is a nuclear enzyme that utilizes NAD as a substrate to transfer up to 100 ADP-ribose groups to a variety of nuclear proteins, including histones as well as PARP itself, following its activation by DNA fragments18. The exact range of functions of PARP has not been established, although the enzyme is thought to play a role in DNA repair12. During major cellular stresses extreme activation of PARP may lead to cell death due to energy depletion1. Thus NAD, the substrate of PARP, is depleted by massive PARP activation, and in efforts to resynthesize NAD, ATP may also be depleted5.

Neural damage following stroke and other neurodegenerative processes is thought to stem from overexcitation attributable to a massive release of the excitatory neurotransmitter glutamate acting on the N-methyl-D-aspartate (NMDA) receptor and other subtype receptors4-6. Evidence includes findings in many animal species that glutamate receptor antagonists block neural damage following vascular stroke and reduce neurotoxicity elicited by treatment of cerebral cortical cultures with glutamate or NMDA (ref. 9). Neurotoxicity elicited by stimulation of NMDA receptors is mediated, at least in part, by augmentation of nitric oxide (NO) formation, as NMDA receptor activation stimulates neuronal NO synthase (nNOS) activity. Protection against NMDA neurotoxicity occurs following treatment of primary brain cultures with NOS inhibitors11,12 and in cortical cultures from mice with targeted disruption of nNOS (ref. 12). Neural damage following vascular stroke is markedly diminished in animals treated with NOS inhibitors13 or in mice with nNOS gene disruption14.

Nitric oxide is a free radical that chemically reacts with its cellular targets. There are multiple potential cellular targets that NO can modify to elicit a range of activities from cellular signaling to cell death. The majority of the toxic effects of NO appears to be a result of the reaction of NO with superoxide to form the very toxic compound peroxynitrite15,16. Both NO and peroxynitrite can cause DNA damage, which activates PARP (ref. 16-18). PARP activation plays a key role in NMDA- and NO-induced neurotoxicity, as PARP inhibitors prevent such toxicity in cortical cultures in proportion to their potencies as inhibitors of this enzyme17.

Studies using drugs that are PARP inhibitors can be criticized on the grounds of lack of specificity and poor bioavailability. Recently Wang et al.18 generated mice with targeted disruption of the gene that encodes PARP (PARP-/-). Tissues from homozygous PARP-deleted mice display a loss of PARP catalytic activity. Although the mice are healthy and fertile and PARP-/- embryonic fibroblasts exhibit normal DNA excision repair, these mice display some abnormalities in cellular proliferation and are susceptible to the development of skin diseases with epidermal hyperplasia18. In the present study we demonstrate that cerebral cortical cultures from PARP-/- mice are completely resistant to neurotoxicity and that tissue damage following vascular stroke is profoundly reduced in PARP-/- animals.
Neurotoxicity in cerebral cortical cultures is reduced in PARP−/− mice

Earlier we showed that the nonselective PARP inhibitors, such as benzamide and 3-aminobenzamide, reduce NMDA toxicity in cortical cultures and also reduce toxicity elicited by NO donors. Benzamide and its derivatives are relatively weak PARP inhibitors with poor bioavailability. At a concentration of 100 μM, benzamide only provides a 50% protection against NMDA neurotoxicity. More recently, 3,4-dihydro-5-[4-(1-piperidinyl)]butoxy]-1(2H)-isoquinolinone (DPO) has been reported to be a much more potent and selective inhibitor of PARP with a 50% inhibitory concentration (IC50) of 40 nM (ref 20). Accordingly, we compared the effects of DPO and benzamide in cortical cultures treated with NMDA or an NO donor, sodium nitroprusside (SNP) (Fig. 1). As reported previously, benzamide provides protection against NMDA- or NO-induced neurotoxicity with a median effective concentration (EC50) of about 100 μM. By contrast, DPO is much more potent providing 50% of maximal protection at 0.2 μM. At concentrations of 10–100 μM, DPO virtually abolishes neurotoxicity, whereas the maximally effective concentration of benzamide only reduces neurotoxicity by 65%. The IC50 of benzamide for PARP is 22 μM (ref 21, 22). Therefore, benzamide is a less potent inhibitor of PARP than DPO; the difference is a factor of 500, which corresponds to the 500-fold difference in potencies of the compounds in protecting against neurotoxicity, fitting with the notion that PARP activation plays a role in neurotoxicity.

We next evaluated toxicity in cortical cultures elicited by NMDA or NO donors or by 60 min of oxygen and glucose deprivation (OGD), comparing cultures from wild-type and PARP−/− mice (Fig. 2). In wild-type cultures, NMDA kills approximately 65% of cells, consistent with previous observations. NMDA-induced neurotoxicity is virtually abolished in cultures from PARP−/− animals and is not significantly different from buffer-treated controls, whereas in heterozygotes (PARP+/−) toxicity is reduced by 72% (Fig. 2a). The NMDA antagonist MK801 abolishes NMDA toxicity in wild-type and heterozygote cultures, but has no effect on the residual minimal toxicity of PARP−/− cultures.

As NO has been shown to mediate the neurotoxic actions of NMDA (ref 10–12), we examined the influence of the NO synthase (NOS) inhibitor nitroarginine on NMDA toxicity. Nitroarginine markedly reduces NMDA toxicity in wild-type cultures and the PARP−/− cultures. Arginine reverses the protective effect of nitroarginine, as previously reported5,12, but has no significant effect in PARP−/− cultures. Neurotoxicity induced by the NO donor, sodium nitroprusside (SNP), is reduced by 73% in PARP−/− cultures and abolished in PARP−/− cultures. The PARP−/− cultures are also resistant to NO neurotoxicity induced by another NO donor, 3-morpholino-sydnonimine hydrochloride (SIN-1). These observations are consistent with the notion that PARP involvement in toxicity is distal to NOS.

We also examined the influence of PARP inhibitors on NMDA neurotoxicity. Benzamide (500 μM) reduces NMDA toxicity in wild-type cultures and PARP−/− with no significant effect on the negligible residual toxicity of PARP−/− preparations. DPO (10 μM) produces greater reduction of NMDA toxicity than benzamide in wild-type and in PARP−/− cultures with no significant effects on PARP−/− preparations. DPO also reduces toxicity elicited by the NO donor, SNP, in both wild-type and PARP−/− cultures with no effect on the PARP−/− cultures. These observations confirm the role of PARP in NMDA-mediated toxicity and demonstrate that the residual toxicity observed in the PARP−/− cultures is due to PARP activity.

The neurotoxicity in cortical cultures elicited by oxygen-glucose deprivation (OGD) has previously been shown to involve NMDA receptor and nNOS activation. In PARP−/− cultures OGD toxicity is virtually abolished and is not significantly different from residual toxicity in buffer-treated control cultures (Fig. 2b). In wild-type cultures and PARP−/− cultures, MK801 and nitroarginine also reduce toxicity with no differential effects in PARP−/− preparations. Arginine reverses the nitroarginine protection in wild-type and PARP−/− culture preparations but not in PARP−/− cultures. Benzamide reduces OGD toxicity in wild-type and PARP−/− cultures preparations to the same extent that it diminishes NMDA toxicity with no influence on PARP−/− preparations. DPO also abolishes OGD toxicity as it does for NMDA toxicity.

Neural damage after vascular stroke is reduced in PARP−/− mice

The combination of oxygen-glucose deprivation and NMDA neurotoxicity in primary neuronal cultures is a common in vitro model for studying early mechanisms of vascular stroke damage. Because of the profound neuroprotection against OGD and NMDA neurotoxicity in this model, we evaluated the extent of brain injury following middle cerebral artery occlusion (MCAo) in the transgenic PARP−/− and PARP−/− mice compared with that in wild-type mice (129/SV). Mice were subjected to 2 hours of MCAo followed by 22 hours of reperfusion, after which the extent of infarct was determined. Infarct volume is reduced by ~80% in PARP−/− animals compared with wild-type animals, 15.4 mm3 versus 69.0 mm3 (Fig. 3a). Significant decreases in injury are evident in each of the five coronal sections except in the most anterior 2 mm (Fig. 3b). Infarct volume in PARP−/− animals is also reduced by ~65% compared with that in wild-type animals.
Fig. 2 Neurotoxicity is profoundly reduced in PARP⁺⁺ cerebral cortical cultures.  
a. A 5-min exposure to 500 μM NMDA kills approximately 65% and 17% of cells in wild-type and PARP⁺⁺, respectively. Toxicity in cultures from PARP⁺⁻ animals exposed to 500 μM NMDA is not significantly different from that in buffer-treated controls. The NMDA antagonist, MK801 (10 μM) abolishes NMDA toxicity in wild-type and heterozygote cultures. Nitroarginine (NArg) (100 μM), a selective inhibitor of NO, reduces NMDA toxicity in wild-type cultures and the PARP⁺⁺ cultures. l-Arginine (LArg) (1 mM) reverses the protection against NMDA toxicity provided by NArg. Benzamide (BZD) (500 μM) reduces NMDA toxicity in wild-type and PARP⁺⁺ cultures. DPQ (10 μM) produces greater reduction of NMDA toxicity than benzamide in wild-type and in PARP⁺⁺ cultures. Neurotoxicity induced by the NO donor, sodium nitroprusside (SNP) (500 μM), is markedly reduced by 73% in PARP⁺⁺ cultures and abolished in PARP⁺⁺ cultures. The PARP⁻⁻ cultures are also resistant to NO neurotoxicity induced by the NO donor, 3-morpholino-sydnonimine hydrochloride (SIN-1) (1 mM). b. 60 min of oxygen-glucose deprivation (OGD) results in 65% cell death in wild-type cortical cultures, which is reduced to 15% in PARP⁺⁺. In PARP⁺⁺ cultures OGD toxicity is virtually abolished and is not significantly different from residual toxicity in buffer-treated control cultures. MK801 (10 μM) and NArg (100 μM) profoundly reduce toxicity in wild-type cultures and PARP⁺⁺ cultures. l-Arginine (1 mM) reverses the protection provided by NArg in wild-type and PARP⁺⁺ cultures. Benzamide (500 μM) reduces OGD toxicity and DPQ (10 μM) abolishes OGD toxicity in wild-type and PARP⁺⁺ cultures.

Each data point is the mean ± s.e.m. (n = 8) of at least two separate experiments. Each data point reflects a minimum of 16,000 neurons counted. Significance was determined by a balanced two-way ANOVA demonstrating significance for the effects of NMDA exposure on genotype (F = 96.359, P < 0.0001) or treatment (F = 18.931, P < 0.0001); or OGD on genotype (F = 406, P < 0.0001) or treatment (F = 191, P < 0.0001) and the Fisher PLSD post hoc test demonstrating differences at *P < 0.0001 when comparing NMDA, SNP, SIN-1-treated or OGD-treated wild-type cultures with PARP⁺⁺ and PARP⁺⁺ cultures (genotype). The Fisher PLSD post hoc test demonstrated differences at †P < 0.0001 when comparing wild-type NMDA-treated or OGD-treated cultures with cultures with the addition of MK801, NArg, BZD or DPQ and differences at ‡P < 0.0001 when comparing NMDA-treated cultures from PARP⁺⁺ animals with MK801, NArg, BZD or DPQ treatments in the PARP⁺⁺ cultures (treatment).

30.1 mm² versus 69.0 mm² (Fig. 3a) with significant protection in the three most caudal sections (Fig. 3b).

In two of the PARP⁺⁺ animals negligible infarct volume was observed, and these animals displayed no neurologic deficit at 22 hours of reperfusion. It is conceivable that these two mice have been completely protected against stroke damage. Alternatively, in these two animals the filament may not have been optimally placed to elicit MCAO. Because of the lack of neurologic deficit at 22 hours, data from these two mice have not been included in analyses of infarct volume (Fig. 3). If data from these two mice were included, the overall infarct volume for the PARP⁺⁺ group would be decreased from 15.4 mm³ ± 1.8 to 12.1 mm³ ± 1.6 (n = 10).

Hemodynamic measures are unaltered in PARP⁺⁺ mice

Hemodynamic alterations can markedly influence the effects of vascular stroke. Accordingly, physiology was monitored in wild-type, PARP⁺⁺ and PARP⁺⁻ animals before, during and after MCAO (Table 1). Regional cerebral blood flow (rCBF) is decreased to approximately 30% of baseline following MCAO and sustained during 2 hours of ischemia similarly among all three groups (Fig. 4). After removal of the filament, rCBF immediately increases to 90–110% of baseline. There are no significant differences in mean arterial blood pressure, core temperature, or blood gases between the groups that could provide nonspecific neuroprotection. Thus, reduction of infarct volume in the PARP⁺⁺ and PARP⁺⁻ animals does not result from altered hemodynamic effects on cerebral blood flow, body temperature, blood gases or blood pressure.

Fig. 3 Protection against focal ischemia in PARP-deficient mice. Infarct volume (a) and area (b) after transient focal ischemia in wild-type (○), PARP⁺⁺ (●) and PARP⁺⁻ (△) mice. Animals were subjected to MCAO filament occlusion for 2 h and reperfused for 22 h as described. The forebrains were sliced into five coronal sections, 2 mm thick, and stained with 1% 2,3,5-triphenyltetrazolium chloride. The volume of infarction was determined by numeric integration of areas of marked pallor with section thickness. Data are presented as the means ± s.e.m. (Control, n = 9; PARP⁺⁺, n = 8; PARP⁺⁻, n = 8). Significance was determined by comparing PARP⁺⁺ or PARP⁺⁻ to wild-type by using a one-way ANOVA with Fisher's post hoc test. *P < 0.05; †P < 0.01; ‡P < 0.001. Additionally, a data point from each individual animal is plotted as a separate point overlayed on each histogram bar.
Table 1  Physiological variables before, during, and after middle cerebral artery occlusion in wild-type (129/SV), PARP−/− or PARP++ mice under halothane anesthesia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Animal</th>
<th>Before</th>
<th>During</th>
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<tr>
<td></td>
<td></td>
<td>(mmHg)</td>
<td>(mmHg)</td>
<td>(mmHg)</td>
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<tr>
<td>MABP</td>
<td>Wild type</td>
<td>86 ± 4</td>
<td>85 ± 5</td>
<td>79 ± 5</td>
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<td>PARP−/−</td>
<td>89 ± 9</td>
<td>86 ± 9</td>
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<td></td>
<td>PARP++</td>
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<td>(°C)</td>
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<tr>
<td>Core temperature</td>
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<td></td>
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<td>PARP++</td>
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<tr>
<td>Arterial PaCO₂ (torr)</td>
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<tr>
<td></td>
<td>PARP++</td>
<td>126 ± 34</td>
<td>116 ± 22</td>
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MABP (mean arterial blood pressure), core temperature, and arterial blood gases (pH, PaCO₂, PaO₂) were measured before occlusion, during ischemia (2 h), and during up to 30 min of reperfusion. Data are presented as the means ± s.d. (wild type, n = 4; PARP−/−, n = 3; PARP++, n = 3). Values were averaged over 15 min before, 2 h during, and 1 h following middle cerebral artery occlusion.

Nevertheless we successfully identified PARP protein and catalytic activity, detectable at modest levels in wild-type mouse forebrain (Fig. 5). PARP protein expression is markedly reduced from wild-type in the PARP−/− forebrain and is not detectable in the PARP++ forebrain (Fig. 5a). PARP catalytic activity is not detectable in the forebrain from PARP−/− or PARP++ mice. We estimate that our PARP assays should have detected as little as 20% of wild-type activity, so that enzyme activity is probably reduced by at least 80% in the PARP++ mice despite a lesser depletion of PARP protein.

**ADP-ribose polymer formation is absent in PARP−/− cortex**

ADP-ribose polymer formation is a marker of PARP catalytic activity. Wild-type mice express high levels of nuclear ADP-ribose polymer formation as identified by immunohistochemistry in the ipsilateral cortex following 2 hours of MCAo and 2 hours of reperfusion (Fig. 6, a and b). There is minimal nuclear staining in the contralateral hemisphere of the same animal. In both the ipsilateral and contralateral cortical hemispheres in the PARP−/− animals following 2 hours of MCAo and 2 hours of reperfusion there is a complete absence of any ADP-ribose polymer staining (Fig. 6, c and d).

**Discussion**

The major finding of this study is the dramatic protection observed in PARP−/− animals against ischemia. In cortical cultures ischemic damage is virtually abolished in neurons derived from PARP−/− animals. NMDA neurotoxicity has been examined in other mice with genetic alterations. In nNOS mice and in transgenic mice overexpressing copper-zinc superoxide dismutase, only about 50–60% maximal protection against NMDA toxicity is observed in the same culture systems as those utilized in the present study. Protection against vascular damage following middle cerebral artery occlusion is also impressive. The 80% reduction in infarct volume of PARP−/− mice exceeds protection reported with any known treatment. Maximal protection of 50–65% typically occurs with glutamate receptor antagonists and NOS inhibitors, treatment with the immunosuppressant FK506 (ref. 27) and after genetic alterations such as in transgenic nNOS mice and mice overexpressing copper-zinc superoxide dismutase. Independently, Moskowitz and associates have also observed pronounced protection against vascular stroke damage in PARP−/− animals. The profound neuroprotection observed in the PARP++ mice, which exceeds protection reported for any other transgenic model or pharmacologic treatment, may represent a common role for PARP activation by other excitotoxic mechanisms in addition to production of free radicals and NO. Additionally, the failure of reported pharmacologic approaches to provide the dramatic protection conferred by the disruption of the PARP gene may reflect issues of bioavailability and drug delivery.

Recently the genetic variation among 129 substrains of mice was reported. Because of the genetic variance between the 129-derived embryonic stem cell lines used for the construction of transgenic animals and the 129 mice available for experimental studies, we cannot exclude the possibility that the dramatic protection we observe in the PARP−/− mice is due to a cosegregating gene from the 129-derived embryonic stem cells. However, reported genetic variability in response to stroke damage between inbred lines of mice is at most 10% (ref. 14). Additionally, we have not observed variability in tissue cultures between strains of mice in vitro in response to NMDA- or NO-mediated neurotoxicity in this or other studies. We routinely use a strain of related 129 mice in our control experiments. Our observations in vitro and in vivo argue against a genetic contribution by the 129-derived embryonic stem cell line in our models of glutamate neurotoxicity and focal ischemia.

The reduction in stroke damage in PARP−/− animals suggests that PARP inhibitors will be useful for treating strokes. This notion is supported by the pronounced protection against NMDA and OGD neurotoxicity afforded by the selective PARP inhibitor DPQ. Greenberg and colleagues have demonstrated that DPQ provides marked protection against neural damage following
Fig. 5 PARP expression and catalytic activity are reduced in PARP-deficient mice. PARP expression (a) and catalytic activity (b) in the mouse brain is markedly reduced in the PARP-/- and PARP-/- mouse brains as compared with wild-type (WT). Protein expression was determined by western blot analysis, and catalytic activity was determined by a self-modification assay. Arrows denote the expression of PARP protein (a) or PARP catalytic activity (b). In the wild-type catalytic assay other ribosylated nuclear proteins present as the grey background, which is reduced with benzamide treatment (WT + Bzd) and is absent in the PARP-/- (+/-) and PARP-/- (-/-) lanes confirming the reduced expression and absence of PARP protein in the brains of these animals. Data shown are representative of duplicate experiments.

vascular stroke in rats.

Reduction in stroke damage and neurotoxicity in PARP-mice is almost as great as in PARP-mice. This protection parallels the greater than 80% reduction in enzyme activity in the PARP-mice preparations. The marked reduction in PARP expression in PARP-mice suggests that more than gene copy number affects the expression of PARP in adult brain. Since PARP is involved in regulating the normal activity of numerous nuclear proteins, it is possible that the loss of one PARP allele significantly affects transcriptional elements that regulate the mature expression of PARP, resulting in lower than expected protein expression.

Activation of PARP may also be important in conditions other than neurotoxicity. For instance, cytokine and oxidant damage to pancreatic islet cells and hepatocytes is reversed by PARP inhibitors and in PARP mice. PARP inhibitors block cardiac damage following occlusion of coronary arteries. Peroxynitrite toxicity to pulmonary epithelium, macrophages and smooth muscle cells is also blocked by PARP inhibitors. Accordingly, PARP inhibitors may be therapeutic in a variety of nonneurologic conditions such as myocardial infarction.

Earlier studies had suggested that PARP activation following DNA damage may be one of several factors contributing to cell death in cerebral ischemia. Consistent with this notion are our observations that ADP-ribose polymer formation is induced in ischemic tissue but not in the contralateral, nonischemic hemisphere of the same animal. Additionally, there is complete ab

sence of ADP-ribose formation in the PARP-/- following MCAo. The present study indicates that PARP activation may be a principal determinant of cell death. In neurotoxicity the following sequence of events presumably takes place (Fig. 7). Ischemia following blood vessel occlusion reduces the resting membrane potential of glia and neurons in the brain. Potassium leaks out of cells and depolarizes neurons leading to a massive release of glutamate. Acting via NMDA receptors, glutamate triggers a release of NO, which combines with superoxide to form peroxynitrite. Peroxynitrite damages DNA and its fragments activate PARP. Massive activation of PARP depletes NAD via ADP-ribose polymer formation. ATP is depleted in an effort to resynthesize NAD leading to cell death by energy depletion. Inhibition of PARP activity spares the cells from energy loss, preventing irreversible depolarization of the neurons and thus provides neuroprotection.

Methods
Primary cortical cultures. Primary cortical cell cultures were prepared from gestational day 16 fetal mice in a procedure modified from that previously described. Briefly, the cortex was dissected, and the cells were dissociated by trituration in modified Eagle’s medium (MEM), 20% horse serum, 25 mM glucose and 2 mM L-glutamine following a 30-min digestion in 0.027% trypsin/saline solution (Gibco BRL, Gaithersburg, MD). The cells are plated on 15-mm multiwell plates coated with polyornithine. Four days after plating, the cells are treated with 5-fluoro-2-deoxyuridine for 3 days to inhibit proliferation of nonneuronal cells. Cells are then maintained in MEM, 10% horse serum, 25 mM glucose and 2 mM L-glutamine in a 1% CO2 humidified 37°C incubator. The growth medium is refreshed twice per week and the neurons are allowed to mature for 14 days in culture before being used for experiments. Ontogeny studies in wild-type cultures have shown that nNOS is expressed at mature levels by day 14 in culture. Mature levels of nNOS neurons correspond to 1–2% of total neuronal population.

Cytotoxicity. The cells were exposed to neurotoxic conditions as previously described. Before exposure, the cells were washed with Tris-buffered control salt solution (CSS) containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 25 mM Tris-HCl (pH 7.4) and 15 mM glucose. The exposure solutions containing experimental reagents were administered for 5 min and then washed off. The cells were then placed in growth medium and returned to the incubator overnight.

Combined oxygen-glucose deprivation was performed as previously described. The culture medium was completely exchanged with deoxygenated, glucose-free Earle’s balanced salt solution (EBSS) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1 mM NaH2PO4 and 0.9 mM CaCl2, bubbled with 5% H2/85% N2/5% CO2. The cultures were kept in an
Fig. 7 A simple model of PARP activation in ischemia. Blood vessel occlusion induces a multitude of cellular events. PARP activation leading to cell death would fit into the known schema as follows: Ischemic events result in the reduction of the resting membrane potential of glia and neurons in the brain. Potassium leaks out of cells and depolarizes neurons leading to a massive release of glutamate. Acting via NMDA receptors, glutamate triggers a release of NO, which combines with superoxide to form peroxynitrite. Peroxynitrite damages DNA, and the fragments activate PARP. Massive activation of PARP leads to ADP-ribosylation and depletion of NAD. ATP is depleted in an effort to resynthesize NAD, leading to cell death by energy depletion.

an aerobic chamber for 60 min containing the gas mixture, 5% H2/85% N2/5% CO2, maintained at 37 °C. Combined oxygen-glucose deprivation was terminated by removal of the cultures from the chamber and replacement of the EBSS solution with oxygenated growth medium. The cultures were returned to a humidified incubator containing 5% CO2 and atmospheric oxygen at 37 °C overnight.

Toxicity was assayed 20–24 h after exposure to cytotoxic conditions by microscopic examination with computer-assisted cell counting following staining of all nuclei with 1 μg/ml Hoechst 33342 stain and staining of dead cell nuclei with 7 μM propidium iodide. Total and dead cells were counted. Gliial nuclei fluoresce at a different intensity than neuronal nuclei and were gated out. Percent cell death was determined as the ratio of live-to-dead cells as compared with the percent cell death in control wells to account for cell death due to mechanical stimulation of the cultures. At least two separate experiments utilizing four separate wells were performed with a minimum of 15,000–20,000 neurons counted per data point. All reagents were purchased from Sigma Chemical Co.

Western blot analysis for PARP. For western blot analysis, fresh mouse brain was homogenized in 20% (wt/vol) 50 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 0.25 M sucrose, 0.2 mM PMSF and 1 mg/ml of chymostatin, leupeptin and pepstatin. The homogenate was centrifuged at 1000g for 15 min at 4 °C. The nuclear fraction, referred to as the pellet, was then washed with the homogenizing buffer. The washed pellet was resuspended in the homogenizing buffer. In a typical PARP assay, each 50 μl mixture contained 0.1 mM [adenylate-32P]NAD (10 Cl/mmol) (NEN Life Sciences, Boston, MA). Following a 5-min incubation at 25 °C, 25 μl of 50% ice-cold TCA was added to each mixture, and the mixture was centrifuged at 8000g for 5 min. The resulting pellet was subjected to two more washes of 50% TCA and two washes of ice-cold acetone. After a brief air-drying, the pellet was dissolved in SDS-PAGE sample buffer containing 4 M urea and was boiled at 90 °C for 15 min. The PARP protein was separated on an 8% SDS-PAGE. The gel was fixed, dried and exposed to a Phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA).

Ischemic model. 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. Adult male, 129/SV mice (Taconic Farms, Germantown, NY) or mutant PARP mice weighing 23–38 g were anesthetized with 1–1.2% halothane in oxygen-enriched air by face mask. The femoral artery was cannulated for measurement of arterial blood gases and blood pressure. Rectal temperature was controlled at or near 37 °C throughout the experiment with heating lamps/water pads in all animals. After baseline arterial blood gas measurement, unilateral MCAo occlusion was performed using intraluminal filament insertion with a 5-0 nylon monofilament. The filament was introduced into the internal carotid artery via the external carotid artery up to a point 6-mm distal to the internal carotid artery–pterygopalatine artery bifurcation. Concurrently, the ipsilateral common carotid artery was occluded by a temporary clip. After 2 h of ischemia, animals were briefly reanesthetized with halothane, and the filament was withdrawn through the external carotid artery, allowing reperfusion of the common and internal carotid arteries but not the external carotid artery.

Determination of infarction volume. After 2 h of ischemia and 22 h of reperfusion, brains were harvested for damage assessment. The forebrain was sliced into five coronal sections, 2 mm thick. These sections were stained with 1% 2,3,5-triphenyltetrazolium, as described. Infarction volume was determined by numeric integration of areas of distinct pallor cross-sectional thickness using digital planimetry.

Immunohistochemical staining for poly(ADP-ribose) polymers. After 2 h of ischemia and 2 h of reperfusion, brains were rapidly removed and flash-frozen. To detect poly(ADP-ribose) polymerase activase, 15-μm-thick sections from fresh frozen brains were incubated in 200 μM NAD at 37 °C for 45 min with modifications. Briefly, sections were fixed in 5% formalin at 20 °C for 10 min and incubated overnight with mouse anti-poly(ADP-ribose) monoclonal antibody (Biornol Research Laboratories, Plymouth Meeting, PA) at a 1:100 dilution. A biotin-SP-conjugated goat anti-mouse IgG (f(ab')2) specific antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was used as secondary at a 1:100 dilution. For signal amplification, an immunoperoxidase ABC kit (Vector Laboratories, Burlingame, CA) was used followed by a TSA-Indirect kit (NEN Life Sciences). Diaminobenzidine was used as a chromogen (Gibco BRL).

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