Intra-mitochondrial Poly(ADP-ribosylation) Contributes to NAD\textsuperscript{+} Depletion and Cell Death Induced by Oxidative Stress∗

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Poly(ADP-ribosylation), primarily via poly(ADP-ribose) polymerase-1 (PARP-1), is a pluripotent cellular process important for maintenance of genomic integrity and RNA transcription in cells. However, during conditions of oxidative stress and energy depletion, poly(ADP-ribosylation) paradoxically contributes to mitochondrial failure and cell death. Although it has been presumed that poly(ADP-ribosylation) within the nucleus mediates this pathologic process, PARP-1 and other poly(ADP-ribosyltransferases) are also localized within mitochondria. To this end, the presence of PARP-1 and poly(ADP-ribosylation) were verified within mitochondrial fractions from primary cortical neurons and fibroblasts. Inhibition of poly(ADP-ribosylation) within the mitochondrial compartment preserved transmembrane potential (ΔΨ\textsubscript{m}), NAD\textsuperscript{+} content, and cellular respiration, prevented release of apoptosis-inducing factor, and reduced neuronal cell death triggered by oxidative stress. Treatment with liposomal NAD\textsuperscript{+} also preserved ΔΨ\textsubscript{m} and cellular respiration during oxidative stress. Furthermore, inhibition of poly(ADP-ribosylation) prevented intranuclear localization of apoptosis-inducing factor and protected neurons from excitotoxic injury; and PARP-1 null fibroblasts were protected from oxidative stress-induced cell death. Collectively these data suggest that poly(ADP-ribosylation) compartmentalized to the mitochondria can be converted from a homeostatic process to a mechanism of cell death when oxidative stress is accompanied by energy depletion. These data implicate intra-mitochondrial poly(ADP-ribosylation) as an important therapeutic target for central nervous system and other diseases associated with oxidative stress and energy failure.

Poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30), the most abundant poly(ADP-ribosyltransferase) in mammalian cells, plays an essential role in excitotoxic neuronal death both in vitro and in vivo (1–4). The presumptive mechanism for this neurotoxic effect involves, sequentially, increases in [Ca\textsuperscript{2+}] via glutamate receptors, activation of nitric-oxide synthase, generation of the free radical peroxynitrite (ONOO\textsuperscript{−}), activation of PARP-1 in response to genomic DNA damage, consumption of NAD\textsuperscript{+} during the formation of poly(ADP-ribose) polymers, and death via energy failure (5). However, the capacity for PARP-1 activation within the nucleus to deplete total cellular energy stores, particularly compartmentalized within mitochondria, remains to be established (4, 6). Because in addition to being abundant in cell nuclei, PARP-1 and other ADP-ribosyltransferases are also prevalent in mitochondria (7–9), where similar to nuclear PARP-1, they facilitate DNA repair in response to oxidative damage (10, 11), we hypothesized that inhibition of mitochondrial poly(ADP-ribosylation) may play a pivotal role in neuronal cell survival under conditions of oxidative stress and excitotoxicity.

Here we show that inhibition of mitochondrial poly(ADP-ribosylation) preserves mitochondrial transmembrane potential (ΔΨ\textsubscript{m}) and NAD\textsuperscript{+} content, maintains cellular respiration, and reduces neuronal cell death triggered by oxidative stress or excitotoxicity. Treatment with liposome-encapsulated NAD\textsuperscript{+} or ATP also preserved ΔΨ\textsubscript{m} and cellular respiration, suggesting that cells can also be rescued by energy repletion after oxidative stress. Our findings suggest that NAD\textsuperscript{+} depletion and energy failure convert poly(ADP-ribosylation) compartmentalized within mitochondria from a homeostatic process to a mechanism of neuronal death, providing a unifying mechanism by which PARP-1 can regulate cell death under conditions of mitochondrial dysfunction, and identifying multiple intracellular targets for inhibitors of poly(ADP-ribosylation). These findings have relevance to both acute and chronic central nervous system diseases where oxidative stress is a contributing factor, including stroke, traumatic brain injury, seizures, and Parkinson’s disease and other neurodegenerative diseases (12, 13).

EXPERIMENTAL PROCEDURES

PARP Activity

PARP enzyme activity was measured using a commercial kit (Trevi- gen, Gaithersburg, MD). Briefly, 1 μg of mitochondrial or nuclear protein extracts were incubated in reaction buffer containing histones and [\textsuperscript{32}P]NAD as exogenous DNA with strand breaks. After incubating at room temperature for 10 min, reactions were terminated and proteins were precipitated with 20% trichloroacetic acid. Incorporation of \textsuperscript{32}P was determined by scintillation counting.

Cell Cultures and Pharmacological Studies

Primary cortical neuron-enriched cultures were prepared from 16 to 17-day-old Sprague-Dawley rat embryos as described (14). Dissociated
cell suspensions were placed in 96-well plates (5 × 10⁴ cells/well) or in plastic dishes coated with poly-d-lysine (1.3 × 10² cells/well). Experiments were performed between 7 and 12 days in vitro.

ONOONO–—Enriched cultures were exposed to 100–250 μM ONOO– (Cayman Chemical, Ann Arbor, MI) in buffer for 30 min, whereon fresh media was replaced.

SIN–1—2 μM SIN-1 (Cayman Chemical) or 20% Me₂SO vehicle in culture media was added to cells in 96-well plates.

Glutamate—Cells were exposed to varying concentrations of l-glutamate with 5 μM glycine (Sigma) in culture media for 10 min. Cultures were pre- or post-treated with either the PARP inhibitor 5-ido-6-amino-7-chloro-2H-pyrido[1,2-a]benzimidazolocarbocyanine iodide (INH2BP) (15, 17) or 20% Me₂SO vehicle, the metalloporphyrin-based peroxynitrite decomposition catalyst FP15 (14) or PBS vehicle, or liposomally encapsulated NAD⁺ or ATP or vehicles (empty liposomes or buffer). Liposomal NAD⁺ and ATP were prepared using a modification of the thin-film method (18). Fibroblasts from PARP-1−/− and corresponding PARP+ mouse embryos (17) were cultured in 96-well plates or in plastic dishes to confluence. For cytotoxicity experiments cells were exposed to 250 μM ONOO– with or without 100 μM INH2BP.

**Immunocytochemistry and Confocal Microscopy**

Neurons grown on poly-d-lysine-coated glass coverslips were fixed for 10 min at 4 °C. Then cells were washed with PBS and permeabilized with 0.2% Triton X-100. Coverslips were incubated for 1 h at 37 °C with primary antibodies against: poly(ADP-ribose) polymers (BD Pharmingen) at optimized dilutions.

**Western Blot Analysis**

Cellular proteins were separated into mitochondrial, nuclear, and cytosolic fractions as described (14), and Western blotting was performed using primary antibodies against: poly(ADP-ribose) polymers (BioMol, Reading, PA), PARP (Cell Signaling, Beverly, MA), the carboxyl terminus of apoptosis-inducing factor (AIF) (Santa Cruz, Santa Cruz, CA), cytochrome c (BD Pharmingen), and cytochrome c oxidase (BD Pharmingen) at optimized dilutions.

**Assessment of ∆Ψₘ**

∆Ψₘ was determined using 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1), a cationic dye that accumulates in mitochondria in a membrane potential-dependent manner (19). Data are presented as a green (488/580 nm)/red (535/610 nm) fluorescence intensity ratio, with an increased ratio representing mitochondrial depolarization. Cells grown on collagen-coated coverslips were incubated in 1 μg/ml JC-1 (Molecular Probes, Eugene, OR) for 20 min at 37 °C. Cells were pretreated with INH2BP, FP-15, or vehicle. The media was replaced with buffer containing ONOO– for 5 min, whereupon the ONOO– was removed and media was replaced. Green/red fluorescent ratios were measured in five predefined fields containing 2–5 cells/field using an Olympus IX70 microscope with a ×60 oil immersion 1.4 numerical aperture optic and an automated stage. Image acquisition and analysis were performed using Simple PCI (Compix, Inc., Cranberry, PA). A minimum of 2 coverslips were imaged per condition.

**Determination of NAD⁺**

Cellular NAD⁺ levels were measured by the enzymatic cycling method using alcohol dehydrogenase (20) with modifications. Cells were homogenized in 0.05 M K phosphate buffer containing 0.1 M nicotinamide (pH 6.0), frozen rapidly, placed in a boiling water bath for 5 min, then cooled in an ice bath for 5 min. Samples were centrifuged for 10 min at 1,000 rpm at 4 °C, then added to a reaction mixture containing 0.065 M glycylglycine, 0.1 M nicotinamide, 0.5 M ethanol, alcohol dehydrogenase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and phenazine methosulfate at pH 7.4. NAD⁺ in the sample generated NADH, which reduces MTT through the intermediate of phenazine methosulfate to formazan. The reaction was allowed to run for 5 min and the absorbance determined at 550 nm. A standard curve was generated using known concentrations of NAD⁺ and NAD⁺ levels were calculated.

**Pulsed Field Gel Electrophoresis**

Pulsed field gel electrophoresis was performed as described (14). Briefly, chromosomal DNA samples were prepared in agarose plugs using a CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad). Fragments were separated on a 1% agarose gel containing 1 μg/ml JC-1 for 20 h. Field strengths were 180 V forward and 120 V reverse, initial and final switching time was set at 5–60 s with a linear ramp. The gel was stained with ethidium bromide and visualized under UV light.

**Isolated Brain Mitochondria**

Adult Sprague-Dawley rat brain mitochondria were isolated by differential centrifugation as described (21). Mitochondrial viability was verified by measuring oxygen consumption. Mitochondria were suspended in a reaction buffer to a final concentration of 1 mg of mitochondrial protein.
drial protein/ml and placed in reaction tubes. Drug or vehicle was added and the mitochondria were placed in a 37°C water bath for 15 min, then 750 μM ONOO⁻/H₂O₂ or pH-adjusted vehicle were added. This concentration of ONOO⁻ was found to completely depolarize mitochondria as determined by Safranin-O. Aliquots of the isolated mitochondrial suspensions were removed at baseline (H₂O₂ 15 min), time 0 and 5 min, centrifuged at 14,000 rpm for 10 min at 4°C, and mitochondrial pellets and supernatants were frozen and stored for batch analysis. For assessment of mitochondrial protein release 20 μl of mitochondria supernatants were dialyzed against PBS in a mini-dialysis device (Pierce). Mitochondrial pellets were homogenized in lysis buffer and handled as described above.

Flow Cytometric Analysis of Cell Death

Following cytotoxicity and drug treatments, cells were harvested using trypsin-EDTA, washed once in ice-cold PBS, and resuspended in 1 ml of Annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). 1 × 10⁶ cells were stained with 5 μl of Annexin V-fluorescein isothiocyanate and 5 μg/ml propidium iodide (PI) in 100 μl of Annexin V binding buffer at 4°C. After 20 min, 400 μl of binding buffer was added to each tube and samples were analyzed using a tri-laser FACS Calibur flow cytometer.

RESULTS

Mitochondrial Poly(ADP-ribosylation) in Fibroblasts and Neurons—We verified that poly(ADP-ribosylation) could be stimulated in mitochondria. PARP-1 was detected in both nuclear and mitochondrial subcellular fractions in fibroblasts from PARP⁻⁻⁻ mice (Fig. 1a). Endogenous PARP activity, measured via incorporation of [³²P]NAD in protein lysates incubated in the absence of nicked-DNA, showed poly(ADP-ribosylation) in both nuclear and mitochondrial cell fractions (Fig. 1b). A lesser degree of baseline PARP activity was detected in nuclear fractions from PARP⁻⁻⁻ fibroblasts, with activity ~20% of that seen in PARP⁺⁺⁺ fibroblasts. Poly(ADP-ribosylation) in control cells suggests that baseline oxidative DNA damage is occurring within both mitochondria and cell nuclei, and is consistent with a previous study (22) showing baseline PARP-1 activity in neurons. Baseline PARP activity was also detected in mitochondrial fractions from PARP⁻⁻⁻ fibroblasts, similarly, activity was ~20% of that seen in PARP⁺⁺⁺ fibroblasts. PARP enzyme activity relative to total protein was greater in mitochondrial compared with nuclear cell fractions (Fig. 1b).

Similar to fibroblasts from wild-type mice, PARP activity was seen in mitochondrial fractions from normal adult rat brain. Mitochondrial and nuclear protein lysates were incubated with and without nicked DNA to activate PARP in the presence or absence of the potent PARP inhibitor INH2BP (6, 15–17). PARP enzyme activity that was inhibited by INH2BP in a dose-dependent manner was detected in both mitochondrial and nuclear fractions (Fig. 1c). A portion of total PARP was previously activated, as protein samples incubated without nicked DNA demonstrated incorporation of [³²P]ADP to levels that were ~40% of total PARP activity. Similar to fibroblasts, PARP activity/μg of protein was greater in mitochondrial compared with nuclear DNA fractions (Fig. 1c).
Mitochondrial Poly(ADP-ribose) Is Inhibited by 5-Iodo-6-amino-1,2-benzopyrone—Consistent with the direct measurement of PARP activity using [32P]NAD, poly(ADP-ribose) polymer formation was more abundant in the mitochondrial versus nuclear protein fraction in control cells (Fig. 2). Thirty min to 2 h after exposure to ONOO⁻, there was a non-significant increase in nuclear poly(ADP-ribosylation) compared with control samples that were not affected by INH2BP. This non-significant increase in nuclear PARP activity in neurons using this dose of ONOO⁻ is similar to reports in fibroblasts, where 500 µM ONOO⁻ is required to stimulate PARP activity (5). In contrast to nuclear poly(ADP-ribosylation), mitochondrial poly-(ADP-ribosylation) was unchanged 30 min to 2 h after exposure to ONOO⁻, however, was significantly reduced by pretreatment with INH2BP versus vehicle (p < 0.001). Multiple poly(ADP-ribosylated) proteins were seen in fractions from each cellular compartment, however, patterns differed with primarily lower molecular weight proteins seen in mitochondrial fractions, compared with −110–140- and 30-kDa bands seen in nuclear fractions. Thus, in neurons using this experimental paradigm, INH2BP reduces basal PARP activity in mitochondria.

Inhibition of Mitochondrial Poly(ADP-ribosylation) Prevents Oxidative Stress-induced Mitochondrial Dysfunction and AIF Release and Neuronal Cell Death—To determine whether 250 µM ONOO⁻ produces mitochondrial dysfunction directly, ∆Ψm, NAD levels, and cellular respiration and were evaluated. Fig. 3a shows that ∆Ψm, determined using the fluorescent dye JC-1 (19), is rapidly lost in neurons exposed to ONOO⁻ and that PARP inhibition attenuates loss of ∆Ψm. Direct quenching of ONOO⁻ using the peroxynitrite decomposition catalyst FP15 (14) also attenuates loss of ∆Ψm (Fig. 3b). PARP inhibition also preserves cellular respiration, as determined by conversion of MTT to formazan (14), in neurons 22 h after exposure to ONOO⁻ versus empty liposomes (Fig. 3c). Pretreatment with liposome-encapsulated NAD⁺ also preserved ∆Ψm versus empty liposomes (Fig. 3f).

We have previously shown that in neurons ONOO⁻ induces nuclear translocation of AIF and large-scale DNA fragmentation, the signature event in AIF-mediated cell death, and that these events can be inhibited by treatment with the ONOO⁻ decomposition catalyst FP15 (14). Similar to FP15, INH2BP completely blocks nuclear translocation of AIF and large-scale DNA fragmentation (Fig. 4, a and b), supporting a role for poly(ADP-ribosylation) in mitochondrial release of AIF and consistent with the report by Yu and colleagues (4, 6). Inhibition or poly(ADP-ribosylation) also prevents egress of cytochrome c from mitochondria to the cytosol after ONOO⁻ exposure.

Fig. 3. Inhibition of mitochondrial poly(ADP-ribosylation) or exogenous NAD or ATP preserves cellular energetics in neurons after oxidative stress. a and b, pretreatment with INH2BP or FP15 preserves ∆Ψm versus vehicle (vehicle, red; drug, blue; INH2BP alone, green; mean ± S.D.). Immunofluorescent images from representative cells for each condition from three independent experiments are shown at 2 h after ONOO⁻ or SIN-1 (n = 12/group for ONOO⁻ and 3/group for SIN-1; *, p < 0.05 versus vehicle). d, treatment with 100 µM INH2BP preserves cellular NAD⁺ content versus vehicle (n = 3–6/group; *, p < 0.05) versus vehicle). e, treatment with liposome encapsulated NAD⁺ (200 µM) or ATP (200 µM), but not empty liposomes or buffer, preserves cellular respiration at 22 h (Lipo, liposomes; n = 3/group; *, p < 0.05 versus vehicle). f, pretreatment with 200 µM liposomal NAD⁺ preserves ∆Ψm versus empty liposomes (empty liposomes, red; liposomal NAD⁺, green; mean ± S.D.). Immunofluorescent images with differential interference contrast from representative cells for each condition are shown in the panels. For c, d, and e, mean ± S.D.; one- or two-way analysis of variance with Tukey post-hoc test.
Neuronal cell death induced by ONOO−, assessed by flow cytometry using PI and Annexin V labeling, is reduced by pretreatment with INH2BP (Fig. 4c). Compared with vehicle treatment, PARP inhibition reduced both PI+/Annexin V− (14 versus 24%, INH2BP versus vehicle, respectively) and PI−/ Annexin V+ (46 versus 27%, INH2BP versus vehicle, respectively) cell profiles. In isolated brain mitochondria (21), a dose of ONOO− (750 μM) that completely depolarizes the mitochondrial membrane initiates rapid release of AIF and cytochrome c (Fig. 5). This dose of ONOO− is relatively high, although it is likely that some proportion of ONOO− was quenched by albumin in the mitochondrial reaction buffer. AIF release, but not cytochrome c release, was inhibited by pretreating isolated viable brain mitochondria with INH2BP. Collectively, these data suggest that inhibition of mitochondrial poly(ADP-riboseylation) preserves Δψm and reduces programmed cell death mediated by AIF.

Inhibition of Poly(ADP-ribosylation) Prevents Excitatory Amino Acid-induced Mitochondrial Dysfunction and AIF Release and Neuronal Cell Death—Because PARP plays a key role in nitric oxide- and glutamate-mediated neuronal death (1, 4), and both mechanisms are felt to involve ONOO−, the cytoprotective effect of INH2BP in neurons treated with glutamate was tested. PARP inhibition preserved cellular respiration after glutamate/glycine exposure (Fig. 6a) and prevented nuclear translocation of AIF (Fig. 6b) compared with vehicle. PARP inhibition reduced both necrotic (1 versus 12%, INH2BP versus vehicle, respectively) and apoptotic (4 versus 14%, INH2BP versus vehicle, respectively) cell death profiles induced by exogenous glutamate/glycine (Fig. 6b). Of note, PARP inhibition led to an increase in Annexin V−/PI− cell profiles (26 versus 1%, INH2BP versus vehicle, respectively) suggesting the potential of delayed cell death, or a shift to an earlier stage of apoptosis in this paradigm.

Mitochondrial PARP-1 and Poly(ADP-ribosylation) in Fibroblasts—Similar to a previous report (17), PARP−/− fibroblasts were less sensitive to ONOO−-induced cytotoxicity. The typical reductions in cellular respiration (MTT), increases in lactate dehydrogenase release, and cell death seen after ONOO− exposure were seen in PARP+/+ but not PARP−/− cells (Fig. 7, a–c). These events were prevented in the case of MTT and lactate dehydrogenase release was determined 2 h after exposure to ONOO−, and inhibited in the case of PI extrusion determined 22 h after exposure to ONOO−, by treatment with INH2BP. These data are consistent with previous reports demonstrating that PARP inhibition reduces necrotic cell death.

PARP-1 Expression and Proteolysis in Neurons and Fibroblasts Exposed to Peroxynitrite—Because PARP-1 cleavage is a feature of caspase-mediated apoptosis (23), PARP-1 expression
of neuron-enriched cultures, or some degree of baseline apoptosis consistent with either apoptosis in dead glial elements typical detected in both the mitochondrial and nuclear compartment, In primary cortical neurons, baseline PARP-1 proteolysis was consistent with necrotic, caspase-independent cell death (Fig. 7). PARP-1 was not detected in PARP-1 nuclear or mitochondrial compartments (Fig. 8). Changes in PARP-1 expression and proteolysis were examined in nuclear and mitochondrial protein fractions. In fibroblasts, changes in PARP-1 expression were not seen after exposure to 250 μM ONOO\(^{-}\) in either the nuclear or mitochondrial compartments (Fig. 8a). As expected, PARP-1 was not detected in PARP-1\(^{-}\) cells. PARP-1 proteolysis was not detected after exposure to 250 μM ONOO\(^{-}\), consistent with necrotic, caspase-independent cell death (Fig. 7). In primary cortical neurons, baseline PARP-1 proteolysis was detected in both the mitochondrial and nuclear compartment, consistent with either apoptosis in dead glial elements typical of neuron-enriched cultures, or some degree of baseline apoptotic neuronal death (Fig. 8b). After exposure to 250 μM ONOO\(^{-}\), PARP-1 cleavage did not appear to be altered, with the exception of the 24-h time point, where a reduction in both PARP-1 and cleaved PARP-1 was seen in both nuclear and mitochondrial fractions, reflecting ONOO\(^{-}\)-induced cell death. Treatment with INH2BP reduced PARP-1 proteolysis induced by ONOO\(^{-}\) in the mitochondrial, and to a lesser degree the nuclear compartment. These data suggest that INH2BP reduces caspase-mediated proteolysis of PARP-1 in both compartments, and are consistent with some degree of caspase-dependent apoptotic cell death in this paradigm (Fig. 4).

**Discussion**

Whereas poly(ADP-ribosylation) contributes to cell homeostasis under basal conditions, pharmacological or genetic inhibition of PARP during conditions of cellular stress, e.g., energy failure or oxidative stress, is beneficial (2, 3, 6). Under these circumstances, it has been proposed that ONOO\(^{-}\) produces genomic DNA damage activating nuclear PARP-1, with subsequent cellular NAD\(^{+}\) depletion, followed by secondary mitochondrial injury, AIF release and nuclear translocation, and cell death (4). However, our data suggest that oxidative stress produces mitochondrial dysfunction directly by promoting rapid loss of ΔΨ\(_{\text{m}}\). Intramitochondrial PARP activity during conditions of limited mitochondrial NAD\(^{+}\) stores related to impaired NAD recycling would exacerbate energy failure by consuming mitochondrial NAD\(^{+}\) directly. In support of this, inhibition of basal mitochondrial poly(ADP-ribosylation) preserves ΔΨ\(_{\text{m}}\), NAD\(^{+}\) levels, and cellular respiration, prevents mitochondrial release of AIF, and attenuates large-scale DNA fragmentation and cell death after oxidative stress. Furthermore, replenishment of NAD\(^{+}\) also preserves ΔΨ\(_{\text{m}}\) and cellular respiration. These data indicate that inhibition of mitochondrial poly(ADP-ribosylation) and energy repletion represent effective strategies to protect cells in the face of insults producing energy failure, and are consistent with the presumption that energy depletion contributes to PARP-related cytotoxicity. PARP inhibition has also been shown to directly protect electron transport chain complexes from inactivation induced by oxidative stress (24). Similarly, NO inhibits mitochondrial metabolism (25).

Compartmentalization of active PARP, NAD\(^{+}\), and DNA damage within mitochondria (7, 8, 11, 26–28) explain the rapid beneficial effects of PARP inhibition after exposure to extramitochondrial ONOO\(^{-}\). In addition, compartmentalization of nitric-oxide synthase and oxygen radicals within mitochondria are a potential source of internally generated ONOO\(^{-}\) (29, 30). Because mitochondrial nitric-oxide synthase is calcium-dependent, elevations in [Ca\(^{2+}\)]\(_{\text{I}}\), occurring under conditions of excitotoxicity could result in both activation of cytosolic and mitochondrial nitric-oxide synthase providing two sources of ONOO\(^{-}\) (31, 32). The results of this study do not discount the importance of nuclear PARP-1 activation in terms of cellular NAD\(^{+}\) consumption; however, it is apparent that mitochondrial poly(ADP-ribosylation) contributes at least in part to the determination of cellular fate under circumstances of NAD\(^{+}\) depletion and energy failure.

Traditionally, PARP-mediated cell death has been felt to be primarily necrotic in nature (1–3, 5, 6). Recently, however, PARP-regulated programmed cell death mediated by AIF has been identified (4). AIF-mediated programmed cell death has many phenotypic features of developmental apoptosis such as DNA fragmentation, phosphatidylserine exposure, and regulation by bcl-2 family proteins; however, important differences also exist, such as large-scale versus oligonucleosomal DNA fragmentation and cell death that progresses despite caspase inhibition (33–36). Both necrotic and AIF-mediated programmed cell death are felt to be caspase-independent, and we have previously reported that the caspase inhibitors N-benzyl-

![Fig. 6. Inhibition of poly(ADP-ribosylation) preserves cellular respiration and attenuates apoptotic and necrotic cell death in neurons after glutamate excitotoxicity.](image-url)
after glutamate exposure cell death was reduced (PI extrusion; Fig. 6c) but a pre-apoptotic phenotype was expressed (PI/H11002/Annexin V binding) in neurons. The concept that preventing apoptosis may switch the mode of cell death to necrosis is not novel (37); however, these data suggest the possibility that preventing necrosis may switch the mode of cell death to apoptosis. This concept, particularly in paradigms that conserve cellular energy stores (38, 39), warrants further study. PARP inhibition also reduced necrosis in fibroblasts exposed to ONOO−/H11002, demonstrating differences not only related to the cytotoxicity paradigm chosen, but also in the cell type used. Differences in response to ONOO− between fibroblasts and neurons may be related to dividing versus non-dividing cells, immortalized versus primary cell cultures, mitochondrial density, or other factors. Nonetheless, taken together these data are consistent with a role for mitochondrial poly(ADP-ribosylation) in caspase-independent cell death.

Whereas activation of PARP during times where cellular energy stores are limited is detrimental (2, 3, 6), it is important to remember that poly(ADP-ribosylation) serves many homeostatic functions as well. In addition to facilitating both genomic and mitochondrial DNA repair (11, 40, 41), poly(ADP-ribosylation) also serves a regulatory role for many transcription factors including nuclear factor-κB, AP-1, and Stat-1 (42), and also...
may regulate transcription itself via loosening chromatin (43). Baseline poly(ADP-ribosylation) within both nuclear and mitochondrial compartments as demonstrated in this study, support such homeostatic functions in cells. In neurons, poly(ADP-ribosylation) may also participate in long-term potentiation (44, 45) and memory formation (46). In dividing cells such as fibroblasts, poly(ADP-ribosylation) via PARP-2 regulates mitosis (47). Collectively these data suggest that poly(ADP-ribosylation) compartmentalized to both mitochondria and nuclei can be converted from a homeostatic process to a mechanism of cell death when oxidative stress is accompanied by energy depletion.

A direct role for mitochondrial poly(ADP-ribosylation) in cell death associated with loss of Δψm, mitochondrial dysfunction, and release of AIF is implicated. These data do not refute previous hypotheses suggesting that overactivation of nuclear PARP-1 consumes total cellular energy stores contributing to mitochondrial dysfunction and cell death. However, these data do represent a paradigm shift, where poly(ADP-ribosylation) compartmentalized within the mitochondria contributes to AIF release and cell death in the face of cellular energy failure.

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