CHAPTER 14

Poly(ADP-Ribose) Polymerase (PARP) and Excitotoxicity

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Abstract

Neuronal injury resulting from glutamate receptor-mediated excitotoxicity has been implicated in a wide spectrum of neurological disorders. Following dramatic results in the preclinical setting, anti-excitotoxic neuroprotective agents have been used in clinical trials for stroke and head injury, but the results have generally been unsuccessful. Hence, alternative targets in the excitotoxic cascade appear to be required. Poly(ADP-ribosyl)ation has been linked to the pathogenesis of numerous disorders of the CNS, including excitotoxicity and ischemic injury. A presumed cascade of glutamate receptor activation leading to excessive free radical formation, DNA damage and then overactivation of PARP-1 is based on studies with drugs that block these various steps. Along this classical view, experiments in our laboratory have shown that the intracellular depletion of ATP and NAD induced by PARP-1 overactivation leads to necrotic cell death in ischemic and excitotoxic models and that PARP-1 inhibitors are protective against necrotic but not apoptotic neuronal death. Therefore, it appears reasonable to propose PARP-1 inhibitors as useful therapeutic agents in pathological brain conditions where necrosis predominates.

Excitotoxicity

Glutamate and related excitatory amino acids are not only responsible for normal synaptic activity and plasticity, but also have an important role in mediating neurotoxic events in the central nervous system. At rest, the concentration of glutamate is <1 mM in the extracellular space, 10 mM in presynaptic terminals, and 100 mM in presynaptic vesicles. These gradients are maintained by electrogenic membrane transporters located on presynaptic terminals of neurons and in glial cells, which recapture excitatory amino acids as soon as they are released in the synaptic cleft. However, if glutamate is released in excess or if energy-dependent uptake systems fail to operate efficiently, the concentration of extracellular glutamate may increase dramatically and become neurotoxic (or excitotoxic). Neuronal injury resulting from glutamate receptor-mediated excitotoxicity has been implicated in a wide spectrum of neurological disease states, including stroke, trauma and epilepsy as well as some types of neurodegenerative or psychiatric disorders. The existence of diseases in the central nervous system in which excitotoxicity is involved may have important clinical consequences, such as the possibility of more effective therapeutic intervention.

The hypothesis that ischemia, hypoglycemia and trauma might share an excitotoxic pathophysiological component is supported by a number of arguments that have been accumulating over the last twenty years (for reviews see refs. 1-5). These include: axon-sparing neuropathology resembling the pattern of neurodegeneration induced by excitotoxic agents, accumulation of extracellular glutamate due to both increased release and/or decreased uptake, alteration in

Poly(ADP-Ribosyl)ation, edited by Alexander Bürkle.

the number and/or subunit composition of glutamate receptors, abnormal and sustained increase in cytosolic free Ca^{2+}, dependence on excitatory afferents and delayed onset of neuronal damage. Probably the most compelling pieces of evidence, however, that implicate excitotoxicity in stroke, trauma and epilepsy are (i) the finding that glutamate receptor antagonists are neuroprotective in a variety of experimental models and (ii) the fact that anti-excitotoxic neuroprotective agents have been and are currently used in clinical trials for stroke and head injury.

Despite the dramatic results in the preclinical setting, phase III clinical trials with anti-excitotoxic drugs have generally been unsuccessful so far.[4,6,7] Several complicating variables have been put forward to explain this discrepancy, including population heterogeneity, morphological and functional differences between human and animal brain, and side-effects of the tested compounds that prevent reaching effective plasma concentrations.[8,9] Fine-tuning in the design of clinical trials, the use of imaging techniques for the evaluation of human brain injury, and the development of more appropriate experimental animal models are among the strategies that need to be utilized in future clinical studies. Also, drugs with a better therapeutic index and aimed at alternative targets in the excitotoxic cascade appear to be required. As our understanding of the pathophysiology of ischemic brain injury and the mechanisms underlying functional recovery increases, newer strategies are emerging and new targets are currently under investigation. For example, alternative approaches to simply blocking glutamate receptors focus on the knowledge gained from studies of postsynaptic signaling pathways[10] and of poly(ADP-ribose) polymerase (PARP) overactivation in excitotoxicity.

PARP-1 and Excitotoxicity: The Suicide Hypothesis

The suicide hypothesis was originally proposed by Berger,[11] who noted that DNA-damaging agents caused PARP-1 activation, a marked depletion of NAD and ATP stores, a pronounced reduction of the energy-dependent processes and finally resulted in necrotic cell death. It was proposed that excessive PARP activation may deplete NAD stores and impair the main NAD^+-dependent metabolic pathways, such as glycolysis and mitochondrial respiration. As a consequence, ATP production is reduced. Furthermore, in an effort to resynthesize NAD^+, the cell consumes a significant amount of ATP (4 molecules of ATP to regenerate one molecule of NAD^+), thus worsening the energetic shortage and contributing to the generation of a vicious cycle leading to cell death (Fig. 1).[12] This mechanism of cell death can be considered nature's way of preventing seriously damaged cells with a high mutation frequency and potential malignant transformation from attempting to repair themselves for survival.

The generation of gene-deficient mouse models by homologous recombination has confirmed the caretaker function of PARP-1 in mammalian cells under genotoxic stress.[13] Unexpectedly, the knockout strategy has also revealed the instrumental role of PARP-1 in cell death after ischemia-reperfusion injury and in various inflammation processes, such as streptozotocin-induced diabetes, arthritis, hemorrhagic shock and chronic colitis.[14] Altogether, these results have established that PARP-1 is a mediator of necrotic cell death by ATP depletion.[15] Recently, it has also been demonstrated that PARP-1 activation is required for translocation of the apoptosis-inducing factor (AIF) from mitochondria to the nucleus.[16] Because this translocation is sufficient to activate apoptosis, PARP-1 appears to be involved in the execution of caspase-independent programmed cell death.

In 1994, it was originally reported that PARP-1 inhibitors significantly reduce glutamate neurotoxicity in cultured cerebellar granule cells.[17] Independent work performed in the same time period showed that NMDA receptor activation and the subsequent influx of Ca^{++} into the neurons may activate nitric oxide synthase (nNOS) and a number of other enzymes, leading to the production of free radical species able to cause DNA strand breaks and pathological activation of PARP-1. These studies demonstrated that in the nervous tissue NO, in addition to its function as a neuronal messenger, may play a major role in glutamate toxicity.[18,19] PARP-1 inhibitors blocked the depletion of both NAD^+ and ATP stores and preserved the energy

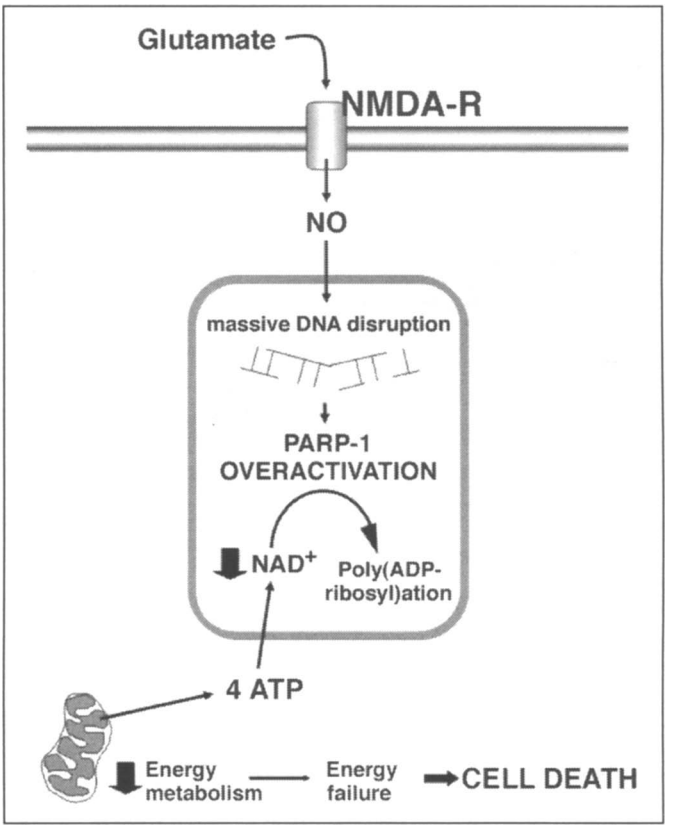

Figure 1. Role of PARP-1 in glutamate-mediated necrosis: a schematic illustration of the "suicide hypothesis". When glutamate stimulates NMDA receptors there is an increase of NO and other reactive oxygen or nitrogen species, which are known to produce DNA strand breaks. When DNA damage is massive, PARP-1 overactivation can lead to cell death of the necrotic type, due to the marked depletion of NAD and ATP tissue stores and the resultant decrease of energy metabolism.

dependent cellular functions. In support of this proposal it was also demonstrated that neurons obtained from nNOS deficient mice are resistant to NMDA toxicity.[14,15] Interestingly, subsequent studies have shown that PARP-1 activation is not involved in the excitotoxic processes activated by other glutamate receptor agonists such as AMPA or kainate.[20]

PARP-1 Inhibitors and Post-Ischemic Neuronal Death

It is widely accepted that the massive release of glutamate and the excessive stimulation of ionotropic and metabotropic glutamate receptors plays a pivotal role in the pathogenesis of neuronal death following cerebral ischemia.[1,2,21-24] A mechanism whereby stimulation of glutamate receptors may lead to neuronal loss involves activation of free radical generating enzymes, formation of reactive species (including NO) and PARP-1 overactivation.[18] The availability of PARP-1$^{-/-}$ mice has enabled a careful evaluation of the link between PARP-1 and stroke. Mixed cortical cell cultures from PARP-1$^{-/-}$ mice are not only resistant to NMDA toxicity, but also to oxygen and glucose deprivation (OGD).[25] In addition, the brain infarct volume after middle cerebral artery occlusion (MCAO) is significantly reduced when performed in PARP-1$^{-/-}$ mice than in wild type animals.[26] Transfection of PARP-1 with a

recombinant virus into the brain of PARP-$1^{-/-}$ mice increased the MCAO-induced infarct volumes, suggesting that PARP-1 is involved in the mechanism leading to neuronal death after stroke.[27] In addition, PARP-1 overactivation, as revealed by poly(ADP-ribose) (PAR) immunoreactivity in focal ischemic animals[26,28] or by PAR cytofluorimetry following OGD in vitro,[29,30] is reduced by treatment with PARP-1 inhibitors. These observations suggest that DNA damage is a crucial initial step in the process leading to cell death after MCAO. Using the comet assay under different experimental conditions to evaluate single and double DNA strand breaks, we demonstrated that single DNA strand breaks are indeed relatively abundant in the rodent cortex and basal ganglia in the first few hours after MCAO. While NMDA receptor antagonists may reduce these breaks, PARP-1 inhibitors decrease infarct volumes without reducing the extent of DNA damage, strongly suggesting that they are working downstream of NMDA receptors and DNA damage.[31]

We have shown that PARP-1 inhibition attenuates neuronal injury in cortical cell cultures[30,32] but not in organotypic hippocampal slices exposed to OGD.[32] We also observed that systemic administration of the PARP-1 inhibitor DPQ (3-10 mg/kg i.p) significantly reduced the infarct volume after MCAO in rats[29] but not CA1 pyramidal cell death following global ischemia in the gerbil.[32] Both necrotic and apoptotic neuronal death have been described following cerebral ischemia.[4,33] In order to understand whether there was a correlation between the results observed with PARP inhibitors and the pattern of cell death in the two different models of post-ischemic brain damage, we examined the necrotic and apoptotic features of OGD-induced cell loss in our in vitro experimental models using biochemical and morphological approaches.

The activation of caspase-3 has a central and exclusive role in the initiation and the execution of neuronal apoptosis.[34] We therefore examined caspase-3-like activity in cortical cultures and organotypic slices exposed to OGD. No significant activation of caspase-3 could be observed at any time point following OGD in cortical cells, suggesting that the involvement of apoptosis in OGD-induced neuronal death in this model is modest. In organotypic hippocampal slices, exposure to OGD led to an increase in caspase-3 activity that was already significant 3 h after the insult and remained stably elevated for up to 24 h, indicating that apoptosis may contribute to OGD-induced neuronal death. Morphological analysis of cortical cells and hippocampal slices exposed to OGD supported this idea. Fluorescence microscopy of cultures stained with the nuclear dye Hoechst 33258 revealed that in cortical cultures virtually all OGD-exposed neurons displayed round, small, and intensely stained nuclei, which is suggestive of necrotic cell death. By constrast, the typical features of apoptotic degeneration, including chromatin condensation and fragmentation were observed in the CA1 region of hippocampal slices.[32] Electron microscopy revealed typical features of necrotic neuronal death in cultured cortical cells examined 6 h after exposure to 60 min OGD. Neurons displayed plasma membrane breaks, large vacuoles as well as swollen mitochondria and organelles in a dispersed cytoplasm. Apoptotic signs of neurodegeneration were found in hippocampal slices exposed to OGD 6 h after insult, when some neurons displayed typical features of apoptosis including clumped chromatin, a darkened cytoplasm with vacuoles and membrane preservation. A significant number of neurons with variable degrees of apoptotic degeneration and apoptotic bodies surrounded by glial cell processes were present 24 h after OGD.[32]

These results are in line with a number of reports showing that reduction of PARP-1 activity with pharmacological or gene targeting strategies does not protect neurons or nonneuronal cells[35] against a variety of deleterious insults leading to caspase activation and apoptosis.[19,36] Because excitotoxicity, which is thought to occur by necrosis[37], is an important component of ischemic neuronal death, a contribution of apoptosis was initially excluded in hypoxia-ischemia. More recently, features of both necrosis and apoptosis have been found in neurons after ischemic insults. Most investigators presently favor the view that programmed cell death and excitotoxicity are triggered in parallel in ischemic tissue, leading to a mosaic of morphological, and even biochemical, features (for reviews see refs. 6,7,38,39). The occurrence or the prevalence of necrotic

or apoptotic cell death in ischemic models depends on a number of factors including the intensity and the duration of the stimulus, the brain region and the type of cell involved, as well as the developmental stage and functional status of the neurons.

These results suggest that PARP-1 overactivation is involved in post-ischemic injury only when necrosis accounts for most of the neuronal death. Similar conclusions have been reached by investigations using fibroblasts[19] or transplanted mesencephalic neurons[40] from PARP-1-deficient mice. A consistent relationship between PARP-1 activation and necrosis but not apoptosis has also been reported for a number of apparently conflicting results obtained in PARP-1 deletion or inhibition studies. For example, pharmacological and genetic inhibition of PARP-1 is neuroprotective in stroke models (for reviews see refs. 14,15), where the massive pan-cellular death of the lesion core is unequivocally necrotic.[41,42] However, morphological features of apoptosis[43] and neuroprotection with interventions that selectively block the apoptotic cascade[44-46] have also been described in focal ischemia, especially in the penumbral area or when the infarction evolves in a delayed fashion. It therefore appears that apoptosis may be revealed under particular conditions in focal ischemia in vivo, similarly to what occurs in cortical cells in vitro, where apoptotic death can be unmasked by extending the OGD exposure to periods longer than 90 min combined with the blockade of the excitotoxic component of OGD-induced neuronal death by ionotropic glutamate receptor antagonists.[47] Interestingly, the brain tissue of mice protected against MCAO by genetic or pharmacological inhibition of PARP-1 displays no change in apoptotic markers as compared with ischemic controls,[26] further suggesting that PARP-1 activation mediates only the necrotic component of focal ischemic injury. We have not examined the necrotic or apoptotic features of CA1 pyramidal cell injury following global ischemia in the gerbil, but previous studies have shown that cell death in this model can coexist as apoptosis,[48,49] necrosis[50] and hybrid forms along an apoptosis-necrosis continuum.[38] Taking our results together with these literature findings, it is tempting to suggest that data obtained in cultured cortical cells may be predictive of events occurring in focal ischemia, whereas OGD exposure in organotypic hippocampal slices may trigger processes that are similar to those evoked in global ischemia.

PARP-1 may be transiently activated in the early phases of apoptosis,[51] but soon thereafter the protein is cleaved and inactivated by caspase-3.[34,52] Recently, a caspase-independent apoptotic pathway triggered by the mitochondrial release of apoptosis-inducing factor (AIF) has been described and a direct relation between PARP-1 activation and this programmed cell death type has been demonstrated.[16] The importance of this pathway in post-ischemic neuronal death remains to be evaluated. Our observations, however, suggest that the pathway may not have a major role in the selective loss of CA1 pyramidal cells observed after transient global ischemia. The process may be active in other pathological situations such as MPP^+-induced degeneration of dopaminergic neurons in the substantia nigra, where PARP-1 inhibitors are highly neuroprotective.[53]

Role of PARP-1 in Models of Mild and Intense NMDA Exposure in Vitro

In a recent study, we characterized two models of NMDA-induced neurotoxicity to investigate the role of PARP-1 and the effects of the PARP-1 inhibitor DPQ in apoptotic and necrotic neuronal death.[54] To this aim, we used mixed cortical cell cultures, containing both glia and neurons, exposed to NMDA under mild or more intense conditions, following the protocol originally described by Bonfoco et al.[55] Addition to the medium of 300 μM NMDA for 10 min (mild insult) or 2 mM NMDA for 20 min (intense insult) led to a time-dependent increase in LDH release which was significant 6 h after the exposure. Mild exposure to NMDA elicited a transient but substantial increase in caspase-3 activity that was maximal at 6 h after the insult, whereas intense NMDA exposure did not elicit a significant activation of caspase-3 at any time point after the insult (Fig. 2). These results were confirmed by morphological analysis of neuronal cultures stained with Hoechst 33258 under fluorescence optics, which

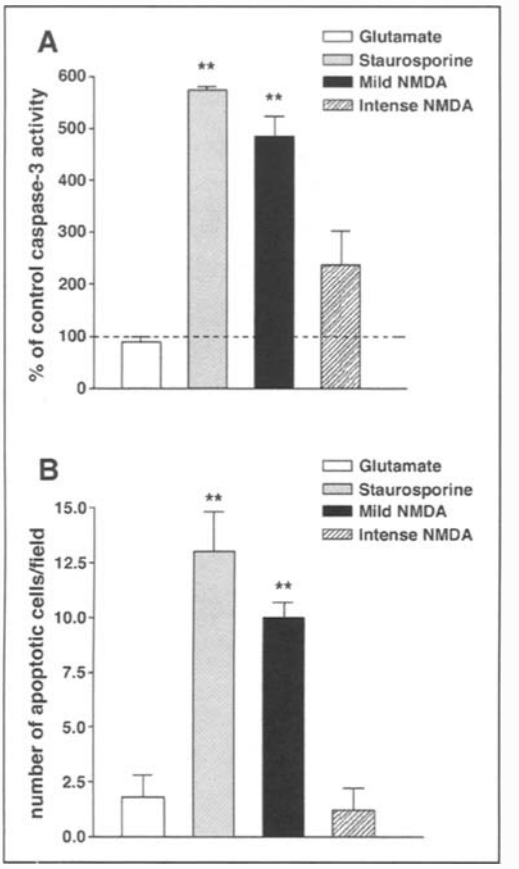

Figure 2. Caspase-3 activity and chromatin fragmentation are increased after 6 h mild but not intense NMDA exposure in murine cortical cells. Cultures were exposed to 1 mM glutamate for 24 h, 100 nM staurosporine for 24 h, 300 μM NMDA for 10 min (mild exposure) or 2 mM NMDA for 20 min (intense exposure). A) Caspase-3 activity was measured in the supernatant of lysed cells at the indicated time after the initiation of exposure to drugs. Data are expressed as percent of caspase-3 basal activity in control cells (16.1 ± 2.5 pmol/10^6 cells) and represent the mean ± SEM of at least 5 experiments. Staurosporine and 300 μM NMDA induced a massive caspase-3 activation, whereas no significant activation was seen with glutamate or 2 mM NMDA. B) Apoptotic nuclei (displaying fragmented chromatin) were revealed by staining cells with Hoechst 33258 at the indicated time after the initiation of exposure to drugs. Apoptotic nuclei were counted in three fields per well under a 40 X lens and expressed as mean number of apoptotic cells per field ± SEM. Staurosporine and 300 μM NMDA induced a significant increase in the number of apoptotic cells, whereas no significant increase was seen with glutamate or 2 mM NMDA. **$p < 0.01$ vs. control (ANOVA + Tukey's w-test).

revealed typical apoptotic condensation and fragmentation of chromatin only when cells were exposed to 100 nM staurosporine for 24 h or 6 h after exposure to 300 μM NMDA for 10 min (Fig. 2). The neuronal content of ATP was significantly reduced at 3 h after mild NMDA exposure, but it returned toward basal levels at later time points. By contrast, a marked and time-dependent ATP depletion was observed following intense NMDA exposure, that reached a 96 ± 4% reduction of basal levels 24 h after the insult. In these models, DPQ reduced neurotoxicity by 84 ± 3% and 50 ± 4% when LDH was measured 6 h and 24 h, respectively, after exposure to 2 mM NMDA for 20 min (Fig. 3A), but was not neuroprotective against exposure

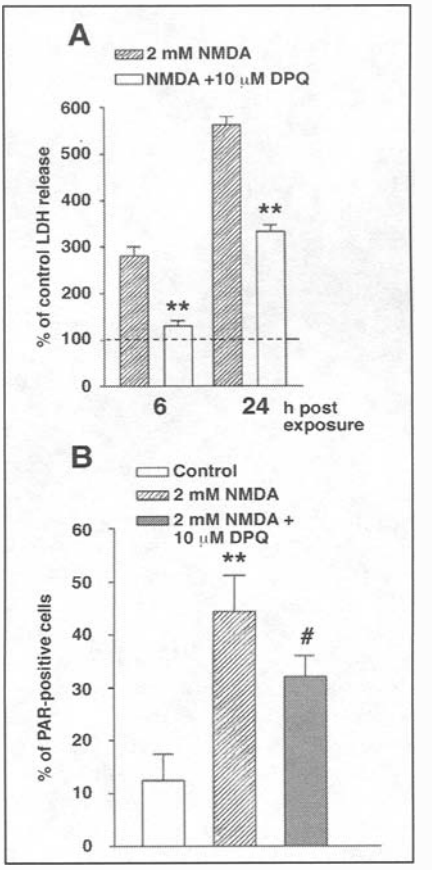

Figure 3. A) The PARP-1 inhibitor DPQ reduces neuronal death following intense NMDA exposure in murine cortical cultures. DPQ (10 μM) was added to the cultures 10 min prior to NMDA exposure, which was performed as indicated in Figure 2. Neuronal death was assessed by measuring the release of LDH in the medium at the indicated time after NMDA exposure. Data are expressed as percent of control LDH release and represent the mean ± SEM of at least 5 experiments. ** $p < 0.01$ vs. NMDA exposure (ANOVA + Tukey's *w*-test). B) DPQ reduces the early activation of PARP-1 induced by intense NMDA exposure in murine cortical cells. One hour after NMDA exposure, cultures were fixed, labeled with antibodies directed against PAR and GFAP, and processed for flow cytometry analysis. The GFAP-positive contribution to PAR fluorescence was subtracted from all samples. The graph shows the percentage of PAR-positive cells in the entire neuronal population, as calculated by counting the relative number of neurons detected at channels for high levels of PAR fluorescence (> 350). Bars represent mean ± SEM of at least four experiments. Intense NMDA exposure elicited the formation of a high intensity PAR fluorescence peak in the neuronal population, indicative of the neosynthesis of PAR, that was prevented by preincubation with DPQ. ** $p < 0.01$ vs. control; # $p < 0.01$ vs, control and $p < 0.05$ vs. NMDA (ANOVA + Tukey's *w*-test).

to 300 μM NMDA for 10 min. The PARP-1 inhibitor was unable to modify caspase-3 activity in cortical cells following mild or intense NMDA insults, except for a slight but significant transient increase in the enzyme activity that was observed 6 h after exposure to 2 mM NMDA. Finally, when we examined the effects of DPQ on the neuronal levels of ATP, the PARP-1 inhibitor had little effect following exposure to 300 μM NMDA or 6 h after exposure to 2 mM NMDA but restored the levels of ATP almost completely (from 4 ± 4% to 72 ± 5% of control levels) 24 h after the intense NMDA exposure.

These results are in line with previous literature data showing that necrotic cell death is prevented by targeted deletion of the PARP-1 gene or by pharmacological inhibition of the enzyme, while apoptosis is unaffected.[19,32] PARP-1 deletion or inhibition have been shown to attenuate cell injury in models in which the type of death is predominantly necrotic, including cerebral[25,26] and myocardial ischemia,[56] and streptozotocin-induced diabetes.[57,58] In contrast, reduction of PARP-1 activity does not protect against killing of hepatocytes by TNF-α with actinomycin D or the death of thymocytes elicited by ceramide, dexamethasone, CD-95, or ionomycin, which are forms of apoptotic cell death.[59] Although PARP-1 activity is also known to affect cell death and survival in a DNA-independent manner via the regulation of transcription factors,[12,60] the finding that DPQ spares ATP in surviving neurons exposed to severe NMDA incubation, as previously demonstrated in other cell types,[61] suggests that in our necrotic model PARP-1 is overactivated and leads to neuronal death through the depletion of NAD and ATP cellular stores (the so-called "suicidal role"). In the milder model, PARP-1 cleavage by caspase-3 activation facilitates apoptosis indirectly by preventing the energy failure induced by PARP-1 overactivation, thereby preserving cellular ATP that is essential for the apoptotic process.

It is interesting to note that 6 h after intense NMDA exposure, DPQ-pretreated cells exhibited a significant increase in caspase-3-like activity that was associated with an increase in the number of apoptotic cells. These data suggest that, as a result of PARP-1 inhibition and the subsequent recovery of ATP levels, there could be a shift in the type of cell death, allowing some cells that would have otherwise died by necrosis to die by apoptosis. In accordance with this view, previous studies have reported that the maintenance of cellular energy levels induced by PARP-1 inhibition after injury permits caspase activation and switches the type of cell death from necrosis to apoptosis.[19,36,62,63] The recovery of energy levels induced by PARP-1 inhibition can also allow the survival of some cells destined to die by apoptosis, as reported in PC12 cells exposed to oxidative damage.[64]

PARP-1 activity increases at early time points after neuronal injury. The formation of PAR in the rat neocortex peaks at 30 min and 2 h after experimental traumatic brain injury before returning to baseline levels.[65] In cortical neurons exposed to NMDA, PAR immunoreactivity can be revealed as early as 15 min following NMDA exposure, with a maximum 1 h later.[16] Therefore, we examined the extent of poly(ADP-ribosyl)ation 1 h after mild or intense NMDA exposure in this study and we observed that the neuronal formation of PAR was increased in a DPQ-sensitive manner in the intense (Fig. 3B) but also in the mild model. These findings confirm that PARP-1 may be transiently activated in the early phases of apoptosis.[51,66,67] Soon afterwards, however, the protein is cleaved and inactivated by caspase-3, thus preventing ATP depletion by PARP-1 overactivation and affording the energy required for the apoptotic active process. This idea is supported by the early but transient reduction in ATP levels following mild NMDA exposure, that was soon restored to levels similar to those observed following prolonged incubation with the pro-apoptotic PKC inhibitor staurosporine.

Concluding Remarks

Excitotoxicity (glutamate-mediated neuronal death) is responsible for numerous neurological and psychiatric conditions that have a high social and financial impact on the society. In several animal models of neurodegenerative diseases, PARP-1 genetic or pharmacological inhibition provides impressive and unparalleled protection, suggesting an importat role for poly(ADP-ribosyl)ation in excitotoxicity. The identification of pathways through which PAR formation fuels excitotoxic neuronal death may increase our understanding of neurodegenerative processes paving the way to innovative therapeutic approaches.

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