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Differential role of poly(ADP-ribose) polymerase-1 in apoptotic and necrotic neuronal death induced by mild or intense NMDA exposure in vitro

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Overactivation of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) plays a key role in the mechanisms responsible for neuronal death. In the present study, we examined the effects of the PARP-1 inhibitor 3,4-dihydro-5-[4-1(1-piperidinyl)buthoxy]-1(2*H*)-isoquinolinone (DPQ) in two models of *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity. The exposure of mixed cultured cortical cells to 300 μM NMDA for 10 min induced a caspase-dependent type of apoptotic neuronal death. Conversely, exposure to 2 mM NMDA for 10 min led to the appearance of morphological features of necrosis, with no increase in caspase-3 activity and depletion in adenosine triphosphate (ATP) levels. DPQ (10 μM) reduced the NMDA-induced PARP activation, restored ATP to near control levels and significantly attenuated neuronal injury only in the severe NMDA exposure model. Similar results were obtained when pure neuronal cortical cultures were used. PARP-1 activation thus appears to play a preferential role in necrotic than in caspase-dependent apoptotic neuronal death.

Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that is primarily activated by DNA strand breaks. Upon activation, the enzyme catalyzes the attachment of chains of poly(ADP-ribose) (PAR) from its substrate β-nicotinamide adenine dinucleotide (NAD) to a variety of nuclear proteins, including PARP-1 itself (Chiarugi, 2002; Herceg and Wang, 2001; Smith, 2001). When DNA damage is mild, PARP-1 is thought to be involved in the maintenance of chromatin integrity. Conversely, overactivation of PARP-1 in response to massive DNA damage has been proposed to play a "suicidal role" due to the marked depletion of NAD and ATP tissue stores and the disruption of oxidative metabolism (Ha and Snyder, 2000; Herceg and Wang, 2001; Szabó and Dawson, 1998). Accordingly, pharmacological PARP-1 inhibition decreases tissue injury in conditions characterized by DNA stress such as neurotoxicity, ischemia-reperfusion injury, diabetes, shock and inflammation (Ha and Snyder, 2000; Szabó and Dawson, 1998; Virag and Szabo, 2002). Moreover, targeted deletion of the PARP-1 gene provides substantial neuroprotection in several experimental models of acute injury (Burkart et al., 1999; Eliasson et al., 1997; Endres et al., 1997; Mandir et al., 1999).

The intracellular depletion of ATP induced by PARP-1 overactivation leads to energy failure and necrotic cell death (Ha and Snyder, 1999; Moroni et al., 2001). In contrast, during the caspase-dependent apoptotic process, PARP-1 is proteolytically cleaved and inactivated by caspase-3, a process that is widely used as an apoptotic marker (Oliver et al., 1998). PARP-1 cleavage in apoptosis is thought to prevent ATP depletion by PARP-1 overactivation, thus affording the energy required for the apoptotic process. Accordingly, the intracellular levels of ATP can regulate the mode of cell death (Eguchi et al., 1997; Leist et al., 1997a): a depletion of ATP, for example, has been demonstrated to shift apoptotic cell death toward a necrotic process (Ha and Snyder, 1999). Recent studies have shown that PARP-1 may also have a role in a caspase-independent pathway of programmed cell death, by promoting the release of apoptosis-inducing factor (AIF) from mitochondria (Cregan et al., 2002; Yu et al., 2002). In this study, we characterized two models of *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity to investigate the role of PARP-1 and the effects of the PARP-1 inhibitor 3,4-dihydro-5-[4-1(1-piperidinyl)buthoxy]-1(2*H*)-isoquinolinone (DPQ) in apoptotic and necrotic neuronal death. To this aim, we used mixed cortical cell cultures, containing both glia and neurons, as well as pure neuronal cortical cultures, exposed to NMDA under mild (10 min at 300 μM) or more intense (20 min at 2 mM) conditions (Bonfoco et al., 1995). We show that mild or intense NMDA exposure elicits apoptotic or necrotic neuronal death, respectively. PARP-1 is activated at an early time point in both models, but PARP-1 inhibition is effective only in reducing necrotic neuronal death.

Abbreviations: DIV, days in vitro; PARP-1, poly(ADP-ribose) polymerase-1; PAR, poly(ADP-ribose); NAD, β-nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; NMDA, *N*-methyl-D-aspartate; DPQ, 3,4-dihydro-5-[4-1(1-piperidinyl)buthoxy]-1(2*H*)-isoquinolinone; LDH, lactate dehydrogenase.

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Results

Assessment of neuronal death following mild or intense NMDA exposure in mixed cortical cell cultures

Mixed cortical cell cultures were evaluated at different time points following NMDA exposure to determine the extent of neuronal death. The release of LDH into the culture medium is a known marker of neuronal injury that under these conditions correlates with phase contrast cell observation and trypan blue staining (Pellegrini-Giampietro et al., 1999a). 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 (Fig. 1). Interestingly, 24 h after the mild NMDA insult, the amount of LDH released was comparable to that observed after adding 100 nM staurosporine to the medium, a well-known apoptotic insult that may lead to secondary necrosis and LDH efflux after prolonged (24 h) exposure in mixed cortical cultures (Koh et al., 1995; Moroni et al., 2001). Neuronal damage induced by the larger NMDA concentration was more extensive, and similar to that observed with 1 mM glutamate for 24 h. This treatment induces necrosis in virtually all neuronal cells but spares the underlying glial cell layer (Moroni et al., 2001; Pellegrini-Giampietro et al., 1999a).

Caspase-3 activity, chromatin fragmentation and ATP levels in mixed cortical cell cultures following mild or intense NMDA exposure

The activation of caspase-3 may have a key role in the initiation and the execution of neuronal apoptosis. To clarify the type of neuronal death in our cultures, we measured the activity of caspase-3 following mild or intense exposure to NMDA and under conditions that are known to induce either necrotic or apoptotic neuronal death in this system (Moroni et al., 2001). Exposure of mixed cortical cultures to an intense excitotoxic and necrotic insult such as 1 mM glutamate for 24 h induced no activation of caspase-3, whereas 24 h exposure to the apoptotic agent staurosporine (100 nM) increased caspase-3 activity up to six times the basal levels (Fig. 2A). Mild exposure to NMDA elicited a transient but substantial increase in caspase-3 activity that was maximal 6 h after the insult (Fig. 2A). In contrast, intense NMDA exposure did not elicit a significant activation of caspase-3 at any time point after the insult. The different effects of mild and intense NMDA exposure were confirmed by morphological analysis of neurons in mixed cultures stained with Hoechst 33258 under fluorescence optics, which revealed typical apoptotic condensation and fragmentation of chromatin only when cells were exposed to 100 nM staurosporine for 24 h (Fig. 2Bb) or 6 h after exposure to 300 μM NMDA for 10 min (Fig. 2Bc). Apoptotic chromatin fragmentation was not observed when neurons were incubated with 2 mM NMDA for 20 min (Fig. 2Bd). Under the latter conditions, most neurons displayed round, small and highly refringent nuclei, which is suggestive of necrotic cell death. A quantitative analysis of chromatin-fragmented nuclei confirmed that the number of apoptotic cells was significantly increased only following exposure to 100 nM staurosporine for 24 or 6 h after exposure to 300 μM NMDA (Fig. 2C), with a pattern that was superimposable to that observed when caspase-3 activity was measured (Fig. 2A).

Because ATP depletion is a feature of necrotic cell death, we also measured the levels of ATP in cultures following mild or intense NMDA exposure. The neuronal content of ATP was significantly reduced 3 h after mild NMDA exposure, but it returned toward basal levels at later time points (Fig. 3). In 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. Glutamate exposure induced a similar dramatic reduction, whereas staurosporine produced only a slight and nonsignificant decrease in ATP levels (Fig. 3).

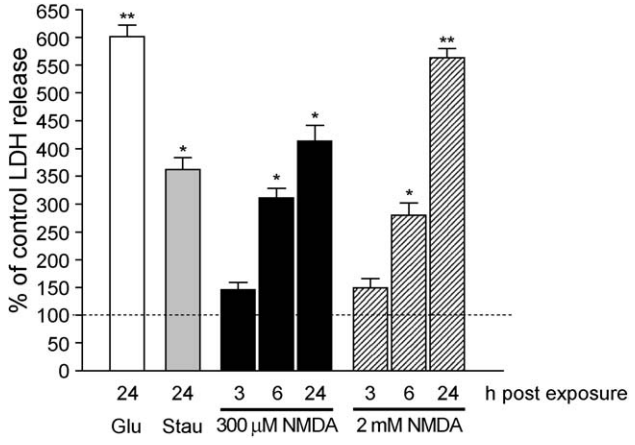

Fig. 1. Neuronal injury induced by mild or intense NMDA exposure in mixed murine cortical cultures. Cultures were exposed to 1 mM glutamate for 24 h, 100 nM staurosporine for 24 h, 300 μM NMDA for 10 min (mild exposure) or 2 mM NMDA for 20 min (intense exposure). Neuronal death was assessed by measuring the release of LDH in the medium at the indicated time after the initiation of exposure. Data are expressed as percentage of control LDH release (31 ± 6 units/l). Background LDH release was determined in control sister cultures and was subtracted from all experimental values. Both mild and intense NMDA exposure induced a time-dependent increase in neuronal injury, whereas the long incubation with the pro-apoptotic agent staurosporine produced a significant LDH efflux that was presumably due to secondary necrosis. *$P < 0.05$ and **$P < 0.01$ vs. control (ANOVA + Tukey's w test).

Effects of the PARP inhibitor DPQ on neuronal death, caspase-3 activity, chromatin fragmentation and ATP levels in mixed cell and pure neuronal cultures following mild or intense NMDA exposure

In this set of experiments, the PARP-1 inhibitor DPQ was added at 10 μM to the incubation medium 10 min before exposing cultured cells to NMDA. In mixed cortical cultures, DPQ reduced neurotoxicity by 84 ± 3% and 50 ± 4% when LDH was measured 6 and 24 h, respectively, after exposure to 2 mM NMDA for 20 min, but was not neuroprotective against exposure to 300 μM NMDA for 10 min (Fig. 4A). Similar results were obtained in pure neuronal cultures. Although the reduction was not significant after 6 h, DPQ reduced neuronal death by 40 ± 6% 24 h after the more severe NMDA insult (Fig. 4B). We then examined the effects of DPQ on caspase-3 activity, as detected 6 and 24 h after NMDA exposure (Table 1). The PARP-1 inhibitor was unable to modify caspase-3 activity in mixed 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

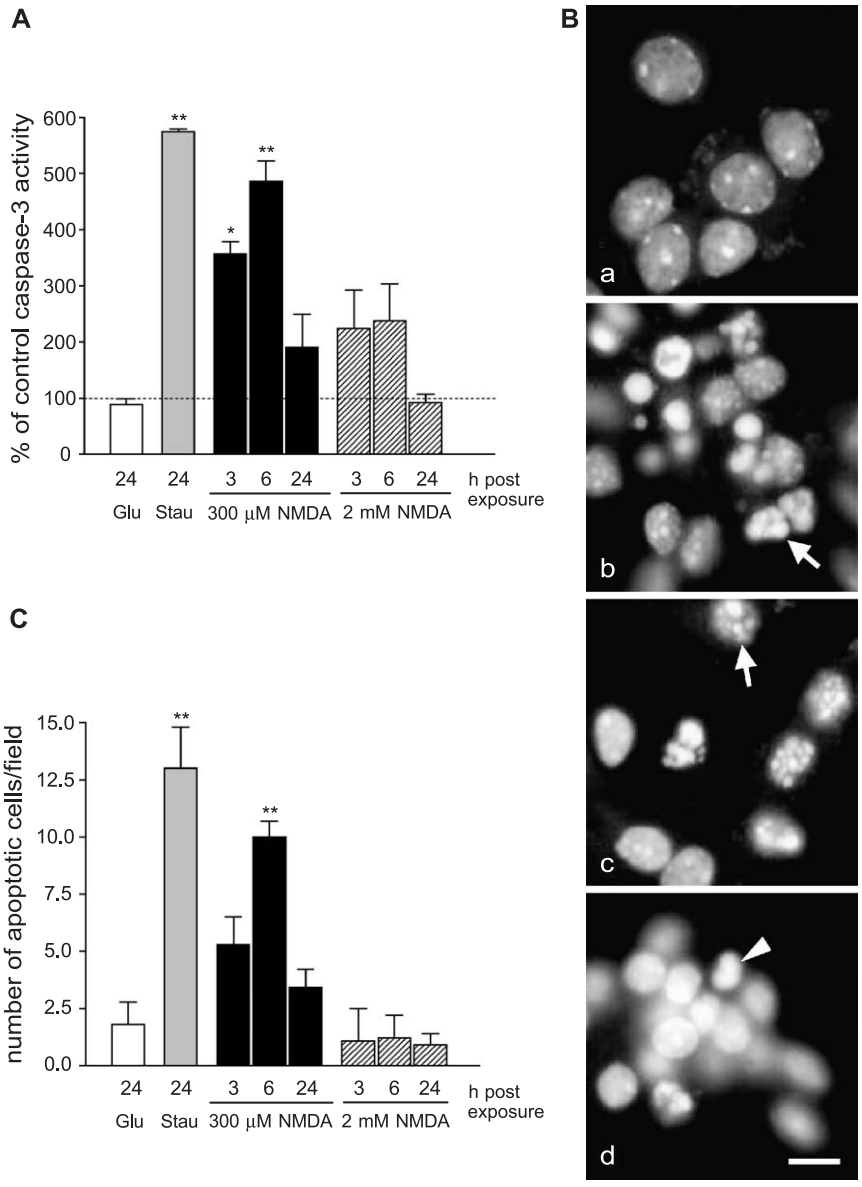

Fig. 2. Caspase-3 activity and chromatin fragmentation are increased after mild but not intense NMDA exposure in mixed murine cortical cells. Cultures were incubated with glutamate, staurosporine or NMDA as indicated in the legend to Fig. 1. (A) Caspase-3 activity was measured in the supernatant of lysed cells at the indicated time after the initiation of exposure to drugs. Data are expressed as percentage of caspase-3 basal activity in control cells (16.1 ± 2.5 pmol/10^6 cells) and represent the mean ± SEM of at least five 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) Cultures were incubated with the nuclear dye Hoechst 33258 at the end of the exposure period and photographed 10 min later under fluorescence optics. (a) Control cultures, displaying dispersed chromatin and evident nucleoli. (b) Neurons incubated with 100 nM staurosporine for 24 h, exhibiting apoptotic nuclear fragmentation (arrow). (c) Six hours following 300 μM NMDA exposure for 10 min, neurons show extensive apoptotic nuclear fragmentation (arrow). (d) Six hours following 2 mM NMDA exposure for 20 min, neurons display mostly small, brilliant and round-shaped nuclei (arrowhead), indicative of necrotic cell death. Scale bar = 15 μm. (C) 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× magnification, averaged and expressed as mean ± SEM number of apoptotic cells per field. 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. *$P < 0.05$ and **$P < 0.01$ vs. control (ANOVA + Tukey's w test).

mM NMDA. Similar findings were obtained by counting the number of chromatin-fragmented nuclei 6 h after exposing the cultures to 2 mM NMDA for 20 min (Table 1). Also, in pure neuronal cultures, DPQ induced a significant increase in caspase-3 activity 6 h after exposure to 2 mM NMDA (Table 2). Finally, when we examined the effects of DPQ on the neuronal levels of ATP in mixed cortical cultures, 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 (Fig. 5A). In pure neuronal cultures, the intracellular content of ATP returned to near control levels (from 32 ±

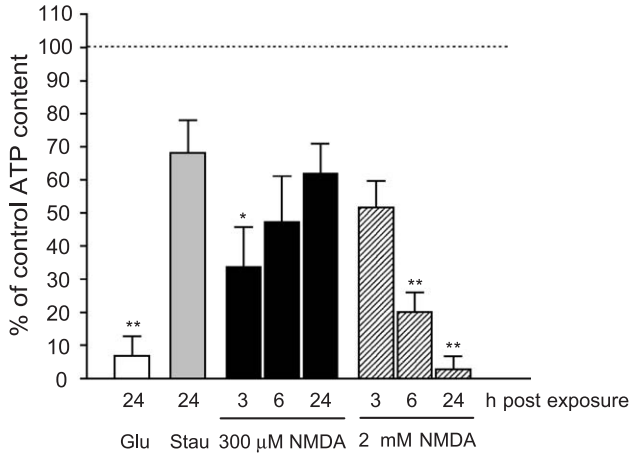

Fig. 3. Intracellular ATP levels in mixed murine cortical neurons following mild or intense exposure to NMDA. Cultures were exposed to glutamate, staurosporine or NMDA as indicated in the legend to Fig. 1. The contents of ATP were evaluated in lysed cells at the indicated time after initiation of exposure to drugs. The glial contribution to energy contents was measured in sister cultures and subtracted to all experimental values. Data are expressed as percentage of basal ATP content in control neurons (1160 ± 90 μmol ATP/10^6 cells). The neuronal levels of ATP were significantly reduced 3 h after mild NMDA exposure and almost completely depleted at 6 and 24 h after intense NMDA exposure. Each bar represents the mean ± SEM of at least six experiments. *$P < 0.05$ and **$P < 0.05$ vs. control (ANOVA + Tukey's *w* test).

6% to 70 ± 6% of basal levels) 24 h after exposure to 2 mM NMDA (Fig. 5B).

DPQ reduces early PARP activation in cultured cortical neurons following intense and mild NMDA exposure

Because PAR immunoreactivity has been shown to be maximal as early as 60 min following NMDA exposure (Yu et al., 2002), we evaluated poly(ADP-ribosyl)ation in neurons present in mixed cortical cultures at this time point following exposure to 300 μM or 2 mM NMDA using a flow-cytometric assay. In control cortical neurons, only a background peak at low levels of relative PAR fluorescence intensity was observed (Fig. 6). Both mild and intense NMDA exposure elicited the appearance of a second peak at higher levels of relative PAR fluorescence intensity, indicative of an increased PARP activity and increased formation of PAR (Fig. 6). This effect was significantly prevented by the addition of 10 μM DPQ to the incubation medium.

Discussion

Our study demonstrates that the presence in the incubation medium of the PARP-1 inhibitor DPQ significantly attenuates neuronal injury in mixed cortical neurons exposed to a more intense but not to a milder NMDA exposure paradigm. Incubation with 2 mM NMDA for 20 min (intense insult) induced mostly necrotic neuronal death in our cultures, whereas exposure to 300 μM NMDA for 10 min (mild insult) displayed morphological and biochemical signs of apoptotic neurodegeneration. Because DPQ was able to reduce the activity of PARP-1 in both systems, these findings suggest that PARP-1 overactivation may be an important mechanism leading to neuronal cell death of the necrotic but not of the caspase-dependent apoptotic type. Because astrocytes may contribute to the neuroprotective effects of DPQ in mixed cortical cultures, we also performed experiments using pure neuronal cultures. The results of these experiments indicate that our observations in mixed cultures primarily reflect activity in cortical neurons.

Necrosis and apoptosis are distinct mechanisms of cell death with very different characteristics (Nicotera and Lipton, 1999; Nicotera et al., 1999; Roy and Sapolsky, 1999). Markers of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation and caspase activation, although a caspase-independent form of apoptosis has recently been described (Cregan et al., 2002; Yu et al., 2002). In this study, we were able to elicit either caspase-dependent apoptosis or necrosis in cultured cortical cells by adjusting the concentrations and the periods of exposure to the excitotoxin NMDA. In a similar fashion, exposure of cortical cultures to relatively short durations or low concentrations of

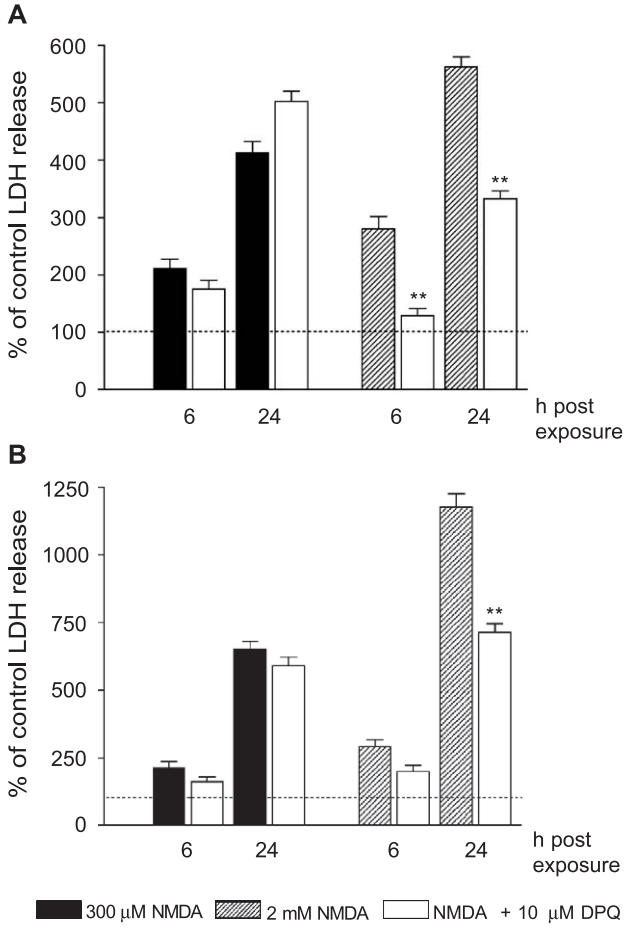

Fig. 4. The PARP-1 inhibitor DPQ reduces neuronal death following intense but not mild NMDA exposure in mixed (A) and pure neuronal (B) murine cortical cultures. DPQ (10 μM) was added to the cultures 10 min before NMDA exposure, which was performed as indicated in Fig. 1. Neuronal death was assessed by measuring the release of LDH in the medium at the indicated time after NMDA exposure. Data are expressed as percentage of control LDH release and represent the mean ± SEM of at least five experiments. In pure neuronal cultures, LDH control release was 18 ± 7 units/l. In both cultures, DPQ significantly decreased neuronal injury only when it was induced by intense NMDA exposure. **$P < 0.01$ vs. NMDA exposure (ANOVA + Tukey's *w* test).

Table 1
Effects of the PARP-1 inhibitor DPQ on caspase-3 activity and chromatin fragmentation in mixed murine cortical cells following mild or intense NMDA exposure

	Caspase-3 activity		Apoptotic cells	
	pmol/10^6 cells	(%)	cells/field	(%)
Control (24 h)	16.1 ± 2.5	(100)	1.2 ± 0.3	(100)
Mild Exposure				
NMDA (6 h)	78.1 ± 9.2**	(485)	10.0 ± 0.7**	(933)
NMDA + DPQ (6 h)	76.3 ± 7.0**	(474)	9.3 ± 1.9**	(775)
NMDA (24 h)	30.6 ± 7.0*	(190)	3.4 ± 0.8*	(283)
NMDA + DPQ (24 h)	36.1 ± 4.7*	(224)	4.2 ± 1.2*	(350)
Intense exposure				
NMDA (6 h)	38.3 ± 15.8*	(238)	1.3 ± 1.0	(108)
NMDA + DPQ (6 h)	56.7 ± 8.2**,#	(352)	5.6 ± 1.1*,#	(466)
NMDA (24 h)	14.8 ± 3.0	(92)	0.9 ± 0.5	(75)
NMDA + DPQ (24 h)	15.3 ± 4.0	(95)	1.5 ± 0.4	(125)

Cultures were treated as indicated in the legend to Fig. 4. Caspase-3 activity was evaluated in lysed cells at the indicated time after NMDA exposure. Apoptotic nuclei displaying fragmented chromatin were revealed as indicated in the legend of Fig. 2C. Data represent the mean ± SEM of at least five experiments. DPQ (10 μM) induced a significant increase in caspase-3 activity and apoptotic neurons 6 h after the intense NMDA insult.
* $P < 0.01$ vs. control.
** $P < 0.05$ vs. control.
# $P < 0.05$ vs. NMDA exposure (ANOVA + Tukey's *w* test).

NMDA was shown to induce delayed neuronal death characterized by apoptotic features, whereas intense exposure to high concentrations of NMDA triggered relatively rapid necrotic cell death (Bonfoco et al., 1995). In another study, glutamate exposure was able to induce either early necrosis or delayed apoptosis depending on the mitochondrial function and cell energy supply (Ankarcrona et al., 1995).

In pure neuronal cultures or in mixed cultures containing neurons and astrocytes, the distinction between apoptosis and necrosis may be difficult because of the poor presence of scavenging cells, such as microglia. Thus, the phagocytic step after apoptosis may not occur and an apoptotic neuron can eventually undergo secondary necrosis and rupture its contents into the surrounding medium (Duke and Cohen, 1986). According to this view, our results show that at late time points, both paradigms of NMDA exposure led to the release of the cytoplasmic enzyme LDH into the medium, indicating cell membrane leakage. Hence, we examined other markers of apoptotic or necrotic cell death in the cultures at various time points after the excitotoxic insult. Our results show that following mild but not intense NMDA exposure, there was a significant increase in the activity of caspase-3, which may have a key role in the initiation and the execution of neuronal apoptosis (Wang, 2000). In confirmation of earlier studies (Bonfoco et al., 1995; Tenneti and Lipton, 2000), the elevation in caspase-3 like protease activity occurred as early as 3 and 6 h after mild NMDA exposure and was associated with an increase in the number of chromatin-fragmented neurons at the same time points. These changes were similar to those observed after prolonged incubation with the pro-apoptotic PKC inhibitor staurosporine. Twenty-four hours after mild NMDA exposure, caspase-3 activity is somewhat reduced while ATP levels are still elevated in cultured neurons. It is possible that apoptotic neurons at this late time point may still possess abundant energy levels but are unable to proceed with the neosynthesis of proteolytic enzymes. Because ATP is required for the progression of apoptosis and its levels are dissipated in necrosis (Leist et al., 1997a), the necrotic nature of cell death following intense NMDA exposure in our cultures was revealed not only by the absence of caspase-3 activation or nuclear fragmentation, but also by the dramatic decrease in the neuronal levels of ATP, which was similar to that observed following incubation with high concentrations of glutamate.

PARP-1 inhibition with DPQ resulted in significant neuroprotection only in the necrotic model of severe NMDA exposure, but had no effect against apoptotic neuronal death following mild 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 (Ha and Snyder, 1999; Moroni et al., 2001). 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 (Eliasson et al., 1997; Endres et al., 1997) and myocardial ischemia (Pieper et al., 2000), and streptozotocin-induced diabetes (Burkart et al., 1999; Pieper et al., 1999). In contrast, reduction of PARP 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 (Leist et al., 1997b). Although PARP-1 activity is also known to affect cell death and survival in a DNA-independent manner via the regulation of transcription factors (Chiarugi, 2002; Ziegler and Oei, 2001), the finding that DPQ spares ATP in surviving neurons exposed to the severe NMDA incubation, as previously demonstrated in other cell types (Lee and Shacter, 1999), 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

Table 2
Effects of the PARP-1 inhibitor DPQ on caspase-3 activity in pure murine cortical neurons following mild or intense NMDA exposure

	Caspase-3 activity	
	pmol/10^6 cells	(%)
Control (24 h)	11.4 ± 1.8	(100)
Mild Exposure		
NMDA (6 h)	29.1 ± 2.8*	(255)
NMDA + DPQ (6 h)	30.3 ± 2.5*	(266)
Intense exposure		
NMDA (6 h)	13.9 ± 3.6	(122)
NMDA + DPQ (6 h)	20.5 ± 2.2*,#	(180)

Pure neuronal cultures were treated as indicated in the legend to Fig. 4. Caspase-3 activity was evaluated in lysed cells 6 h after NMDA exposure. Data represent the mean ± SEM of at least three experiments. DPQ (10 μM) induced a significant increase in caspase-3 activity 6 h after the intense NMDA insult.
* $P < 0.01$ vs. control.
# $P < 0.05$ vs. NMDA exposure (ANOVA + Tukey's *w* test).

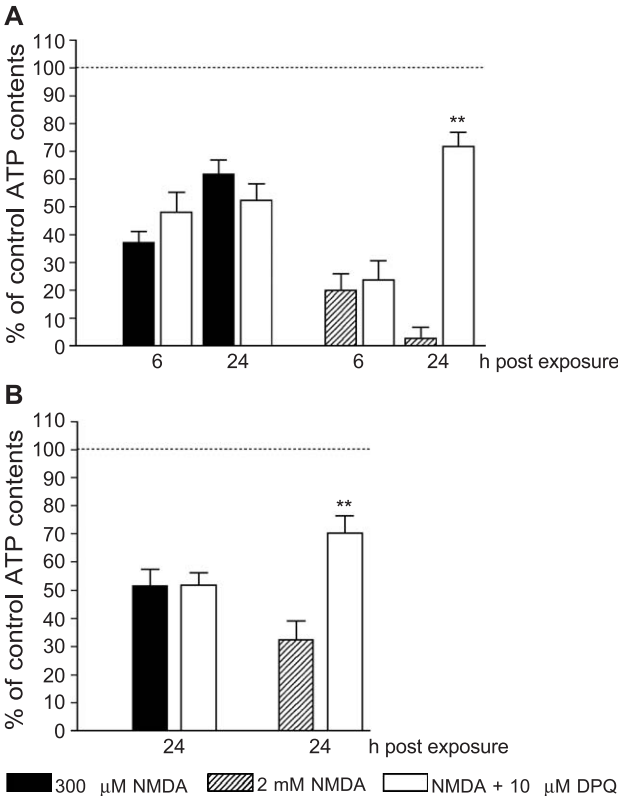

Fig. 5. The PARP-1 inhibitor DPQ recovers ATP levels in mixed (A) and pure neuronal (B) murine cortical cultures following intense but not mild NMDA exposure. Cultures were treated as indicated in the legend to Fig. 4. ATP levels were evaluated in lysed cells at the indicated time after NMDA exposure. Data are expressed as percentage of basal ATP content in control neurons and represent the mean ± SEM of at least five experiments. In pure neuronal cultures, ATP basal levels were 980 ± 110 μmol ATP/10^6 cells. In both cultures, DPQ almost completely prevented the reduction of ATP observed 24 h after an intense NMDA insult. $^{**}P < 0.01$ vs. NMDA exposure (ANOVA + Tukey's *w* test).

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. According to 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 (Eguchi et al., 1997; Ha and Snyder, 1999; Leist et al., 1997a; Walisser and Thies, 1999). 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 (Cole and Perez-Polo, 2002).

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 (LaPlaca et al., 1999). 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 (Yu et al., 2002). 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 both models. These findings confirm that PARP-1 may be transiently activated in the early phases of apoptosis (Boulares et al., 1999; Simbulan-Rosenthal et al., 1998; Scovassi and Poirier, 1999). Soon after, 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 (Scovassi and Poirier, 1999). This idea is supported by the early but transient reduction in ATP levels following mild NMDA exposure, that was soon after restored to levels similar to those observed following prolonged incubation with the pro-apoptotic PKC inhibitor staurosporine.

In conclusion, our results show that PARP-1 is activated at an early time point in both models, but contributes significantly to the necrotic cell death pattern observed after intense NMDA exposure but not to the processes leading to apoptotic cell death following mild NMDA exposure. These findings suggest that PARP-1 inhibitors may be of therapeutic importance in brain pathologies where necrosis predominates.

Experimental methods

Materials

NMDA and DPQ were purchased from Tocris Cookson (Bristol, UK). Tissue culture reagents were obtained from GIBCO-BRL (San Giuliano Milanese, MI, Italy) and ICN Pharmaceuticals (Opera, MI, Italy). Lactate dehydrogenase (LDH) activity was measured using the Cytotoxicity Detection Kit (LDH) from Roche Italia (Monza, MI, Italy). Hoechst 33258 (bis-benzimide), propidium iodide (PI), the EnzChek™ Caspase-3 Assay Kit #2, the ATP Determination Kit, the anti-glial fibrillary acidic protein (GFAP) fluorescein isothiocyanate (FITC)-conjugated antibody and the phycoerythrin-conjugated goat anti-rabbit IgG were purchased from Molecular Probes Europe (Leiden, The Netherlands). Tergitol (NP-40) was from Sigma-Aldrich (Milan, Italy). The polyclonal antibody to PAR (LP96-10) was from Alexis Corp. Italia (Vinci, FI, Italy).

NMDA neurotoxicity in cortical cell cultures

Cultures of mixed cortical cells containing both neuronal and glial elements were prepared as previously described in detail (Pellegrini-Giampietro et al., 1999a,b), used at 14 days in vitro (DIV) and exposed at room temperature to 300 μM NMDA for 10 min (mild insult) or to 2 mM NMDA for 20 min (intense insult). During NMDA exposure, the original culture medium [Eagle's Minimal Essential Medium (MEM, with Earle's salts, glutamine- and $NaHCO_3$-free) supplemented with 38 mM $NaHCO_3$, 22 mM glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% heat-inactivated horse serum and 10% fetal bovine serum] was replaced by thorough exchange with a HEPES controlled salt solution (HCSS, composition in mM: 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 20 HEPES, 15 glucose, 10 NaOH and 10 mg/l phenol red). After the exposure to NMDA, cultures were rinsed with HCSS, the original medium was restored and cells were kept at 37°C, 100% humidity and 95% air/5% CO_2 atmosphere until neurodegeneration was assessed. To achieve a maximal degree of neuronal injury in this system, mixed cortical cultures

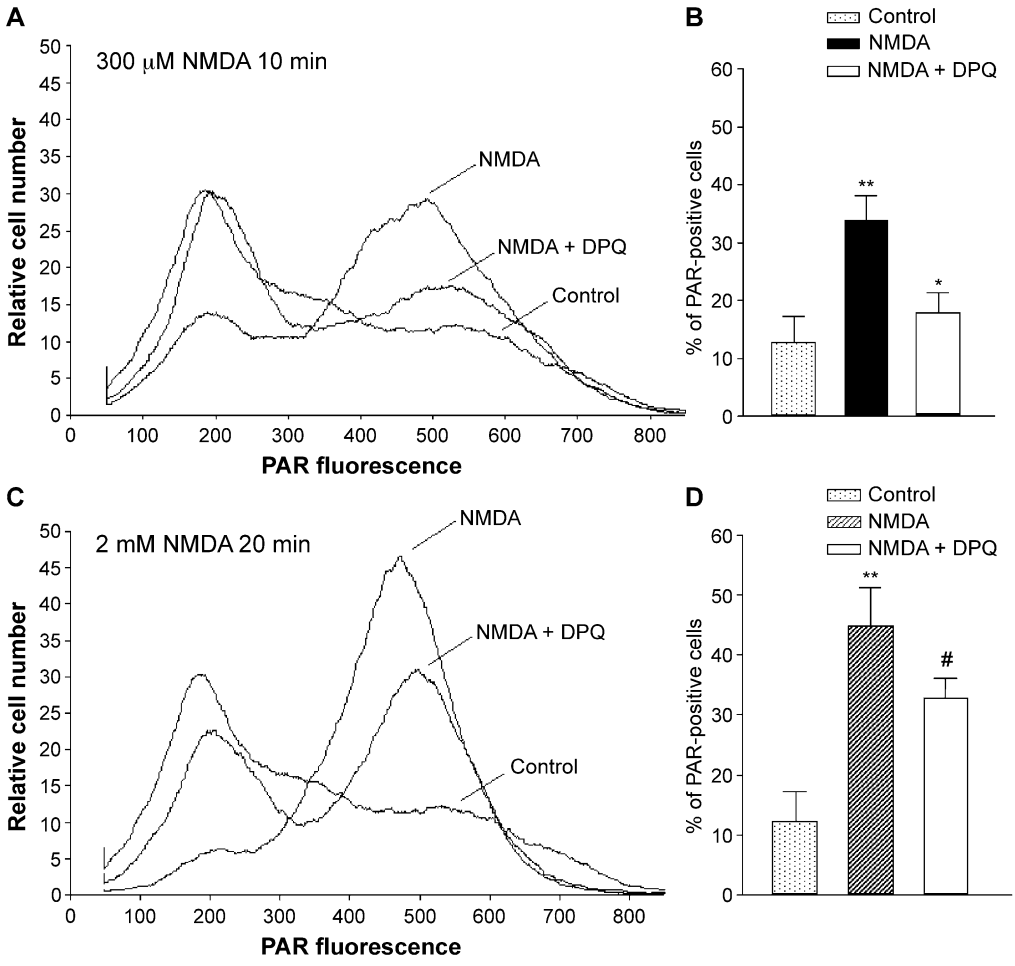

Fig. 6. DPQ reduces the early activation of PARP induced by intense and mild NMDA exposure in mixed murine cortical cells. Cultures were treated as indicated in Fig. 4. One hour after NMDA exposure, cultures were fixed, labeled with antibodies directed against PAR and GFAP, and processed for flow cytometry analysis. The GFAP-positive contribution to PAR fluorescence was subtracted from all samples. The graphs A and C plot the relative number of cells displaying increasing levels of PAR fluorescence and are representative of four analyses. B and D show 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. Both mild and intense NMDA exposure elicited the formation of a high intensity PAR fluorescence peak in the neuronal population, indicative of the neosynthesis of PAR, which was prevented by preincubation with DPQ. *$P < 0.05$ and **$P < 0.01$ vs. control; #$P < 0.01$ vs. control and $P < 0.05$ vs. NMDA challenge (ANOVA + Tukey's w test).

were exposed for 24 h to a high concentration (1 mM) of glutamate.

Pure neuronal cultures were prepared by seeding cortical cells (resuspended in Neurobasal™ medium with B-27 supplement, GIBCO) onto poly-D-lysine-coated wells, used at 8–12 DIV and exposed to mild or intense NMDA insult as described for mixed cultures.

Cell damage was quantitatively evaluated in both cultures by measuring the amount of LDH released from injured cells into the extracellular fluid 24 h following exposure to NMDA, as previously described (Pellegrini-Giampietro et al., 1999a,b). Background LDH release was determined in control cultures not exposed to NMDA and was subtracted from all experimental values. The resulting value correlated linearly with the degree of cell loss estimated by observation of cultures under phase-contrast microscopy or under bright-field optics following 5 min incubation with 0.4% trypan blue, which stains debris and nonviable cells.

Measurement of caspase-3-like protease activity

The EnzChek™ Caspase-3 Assay Kit # 2 was used to measure increases in caspase-3 and other Asp-Glu-Val-Asp (DEVD)-specific protease activities in cortical cultures. The assay is based on the use of a bisamide derivative of rhodamine 110, containing DEVD peptides covalently linked to each of the dye amino groups, thereby suppressing its fluorescence. Upon enzymatic cleavage, the nonfluorescent bisamide substrate is converted in a two-step process to rhodamine 110, which exhibits fluorescent spectral properties similar to those of fluorescein. Mixed cell or pure neuronal cortical cultures were harvested and washed in phosphate-buffered saline. After brief centrifugation, cell pellets were resuspended in lysis buffer (Component C) and incubated at 4°C for 30 min. Lysed cells were then centrifuged (5 min at 5000 rpm) and the supernatants used to detect caspase-3 proteolytic activity. The assay was performed by adding 50 μl of reaction buffer (Component D, final concentra-

tion: 10 mM PIPES, pH 7.4, 2 mM EDTA, 5 mM dithiothreitol, 0.1% CHAPS and 50 μM benzyloxycarbonyl-DEVD-rhodamine 110 substrate) to 50 μl of the supernatants. After incubating the samples at room temperature for 30 min, fluorescence was measured with a fluorometer set at 496 nm excitation and 520 nm emission. Data were calculated as picomoles of rhodamine 110/10^6 cortical cells.

Microscopic analysis of cell necrosis and apoptosis

Nuclear morphological features of necrotic and apoptotic degeneration were analyzed by fluorescence microscopy with the nuclear dye Hoechst 33258 (bis-benzimide). Mixed cortical cells were fixed with 4% paraformaldehyde for 30 min, washed in phosphate-buffered saline and then incubated for 10 min at 37°C with 1 μg/ml Hoechst 33258. After washing in phosphate-buffered saline (PBS), cells were viewed for nuclear chromatin morphology in an Olympus IX50 fluorescence inverted microscope equipped with an Olympus WU filter set (excitation: 330–385 nm) and a 40× or 60× objective. Normal cells were identified by intact round-shaped nuclei with diffuse fluorescence and necrotic cells by highly refringent smaller nuclei with uniformly dispersed chromatin. Because nuclear condensation is a feature of both necrosis and apoptosis, only cells displaying nuclear fragmentation and blebbing were considered apoptotic. Fragmented apoptotic nuclei were counted in three fields per well under a 40× magnification, averaged and expressed as mean number of apoptotic cells per field of at least five wells.

Measurement of ATP contents

Intracellular ATP was extracted from cells of mixed or pure neuronal cortical cultures and measured by a luciferin-luciferase bioluminescence assay (ATP Determination Kit). The assay is based on luciferase's requirement for ATP in producing light from the reaction with luciferin. The whole cell population, including glial cells in mixed cortical cultures, was subjected to the assay. Cells were washed and collected in ice-cold PBS: 10 μl of the cell lysate was added to 90 μl of working buffer containing 0.5 mM luciferin, 1.25 μg/ml luciferase, 25 mM Tricine buffer (pH 7.8), 5 mM $MgSO_4$, 100 μM EDTA and 1 mM DTT. Luminescence was analyzed after a 3-s delay with a luminometer (LKB-Wallac, Turku, Finland). A standard curve was generated from known concentrations of ATP in each experiment and used to calculate the concentration of ATP in each sample. The glial contribution to energy contents in mixed cortical cultures was evaluated in sister cultures and subtracted to all samples. Data were calculated as micromoles of ATP/10^6 cortical cells.

Measurement of PARP activity by flow cytometry

PARP activity was evaluated in mixed cortical cells exposed to NMDA by cytofluorimetric measurement of PAR formation according to Affar et al. (1999), with minor modifications. One hour after NMDA exposure, neuronal cultures were detached using 0.05% trypsin for 5 min at 37°C in PBS, washed with cold PBS and fixed with 4% paraformaldehyde at room temperature. Fixed cells were permeabilized with 0.2% NP-40, washed with PBS, saturated with PBS-MT (PBS containing 5% nonfat powdered milk and 0.1% Tween 20) for 1 h and then incubated overnight at 4°C with anti-PAR (1:100) and FITC-conjugated anti-GFAP (1:100) antibodies diluted in PBS-MT. After several washes with PBS-MT, cells were incubated with a phycoerythrin-conjugated anti-rabbit IgG (1:50) for 30 min. The cell suspension (containing both neurons and astrocytes) was analyzed using a flow cytometer (Coulter XL, Coulter Cytometry, Hialeah, FL, USA). By means of appropriate electronic gates, neuron-related events (PAR positive neurons) were sorted by subtracting FITC-labeled cells (GFAP-positive astrocytes) from phycoerythrin-positive cells.

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