Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-d-aspartate or nitric oxide/superoxide in cortical cell cultures

(Excitotoxicity/cortical neurons/peroxynitrite)

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ABSTRACT N-Methyl-d-aspartate (NMDA) receptor-mediated neurotoxicity may depend, in part, on the generation of nitric oxide (NO) and superoxide anion (O2−), which react to form peroxynitrite (OONO−). This form of neurotoxicity is thought to contribute to a final common pathway of injury in a wide variety of acute and chronic neurologic disorders, including focal ischemia, trauma, epilepsy, Huntington disease, Alzheimer disease, amyotrophic lateral sclerosis, AIDS dementia, and other neurodegenerative diseases. Here, we report that exposure of cortical neurons to relatively short durations or low concentrations of NMDA, S-nitrosocysteine, or 3-morpholinosydnonimine, which generate low levels of peroxynitrite, induces a delayed form of neurotoxicity precipitated by apoptotic features. Pretreatment with superoxide dismutase and catalase to scavenge O2− partially prevents the apoptotic process triggered by S-nitrosocysteine or 3-morpholinosydnonimine. In contrast, intense exposure to high concentrations of NMDA or peroxynitrite induces necrotic cell damage characterized by acute swelling and lysis, which cannot be ameliorated by superoxide dismutase and catalase. Thus, depending on the intensity of the initial insult, NMDA or nitric oxide/superoxide can result in either apoptotic or necrotic neuronal cell damage.

Oxidative stress, leading to the formation of free radicals, has been implicated in a final common pathway for neurotoxicity in a wide variety of acute and chronic neurologic diseases (1). Excessive stimulation of excitatory amino acid receptors in these disorders may trigger the production of free radicals. In particular, neurotoxicity associated with overstimulation of N-methyl-d-aspartate (NMDA) receptors is thought to be mediated by an excessive Ca2+ influx, leading to a series of potentially toxic events (1). One of these events is the activation of nitric oxide synthase and the subsequent production of nitric oxide (NO) (2). Another event is the stimulation of phospholipase A2 or Ca2+ overload of mitochondria, leading to the generation of superoxide anion (O2−) (3). NO can react with O2− to form peroxynitrite (OONO−) (4), which results in dose-dependent neuronal damage (5).

The mode of neuronal cell death after damage initiated by excitotoxins or free radicals, however, has remained controversial, with some groups finding features of necrosis but others reporting characteristics of apoptosis (6–9). Although similar factors might trigger either phenomenon, this does not mean that the nature of the two forms of cell death is the same.

Necrosis and apoptosis are distinct mechanisms of cell death with very different characteristics. Necrosis is caused by catastrophic toxic or traumatic events with passive cell swelling, injury to cytoplasmic organelles including mitochondria, and rapid collapse of internal homeostasis. Necrosis leads to membrane lysis, release of cellular contents, and resulting inflammation (10, 11). In contrast, apoptosis is an active process of neuronal cell destruction with specific defining morphologic and molecular features, the description of which is still evolving. Apoptosis is characterized by cell shrinkage, membrane blebbing, and, if a nucleus is present, nuclear pyknosis, chromatin condensation, and genomic fragmentation (12, 13). A major difference between the two types of cell death is the generalized involvement of neighboring cells within necrotic tissue. To prevent leakage of excitatory amino acids, proteolytic enzymes, DNA, and oxidized lipids with a proinflammatory response, apoptotic cells condense their chromatin, shrink, and in some cases shield their intracellular milieu by cross-linking membrane proteins. In a sense, apoptosis represents, at a cellular level, “death with dignity”—the internal and external membranes are preserved so that the cellular contents are safely sealed within the dying cells until phagocytes intervene.

In attempting to define the mechanism and events leading to these different forms of neuronal cell death, it is extremely important to be aware of an artifact that can occur in tissue culture models—the distinction between apoptosis and necrosis can be confused because of the lack of scavenging cells, and thus the phagocytic step after apoptosis may not occur. Instead, an apoptotic cell can eventually undergo secondary necrosis and rupture its contents into the surrounding medium (14). Thus, it is mandatory to look for features of apoptosis at various time points after the insult and not to unduly delay these observations, as secondary necrosis may intervene and therefore obfuscate the true nature of the injury to the compromised cell. In vivo, a similar situation may ensue when the number of apoptotic cells overwhelms the ability of the tissue to remove them. In this case, a late necrosis may develop in the tissue. It is true that not all forms of apoptosis are similar, and probably further classifications will be required. However, most authorities believe that apoptosis is an active form of cell death, entirely distinct from necrosis. In the latter, a general failure of cellular homeostasis results in energy loss and often uncontrolled Ca2+ overload, rapidly resulting in cell blebbing and lysis.

We noted for neurons that apoptosis may take some time to develop after the inciting event, whereas massive necrosis could sometimes be observed within minutes or an hour. Therefore, we wondered whether the intensity of the original insult could be related, at least in some cases, to the pathway

Abbreviations: LDH, lactate dehydrogenase; NMDA, N-methyl-d-aspartate; SNOC, S-nitrosocysteine; SOD, superoxide dismutase; TUNEL technique, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling; SIN-1, 3-morpholinosydnonimine.

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to neuronal cell death? In this study we show that mild excitotoxic or free radical insults result most often in apoptotic neuronal cell death, whereas necrosis predominates after intense, fulminate insults.

**MATERIALS AND METHODS**

**Generation of NO\(^{-}\), O\(_2^-\), and OONO\(^{-}\).** S-Nitrosocysteine (SNOC) and peroxynitrite (OONO\(^{-}\)) were prepared as we have described (5, 15). 3-Morpholinosydnonimine (SN-1; 1 mM) has been shown to produce NO\(^{-}\) and O\(_2^-\), resulting in OONO\(^{-}\) formation (16). With the short half-life of OONO\(^{-}\), its steady-state concentration generated by SN-1 should only approach low micromolar levels in our culture system. SNOC (200 \(\mu\)M) decomposes homolytically to yield NO\(^{-}\) at a relatively rapid rate (\(1/2 < 1\) min), but only a low micromolar level of NO\(^{-}\) is produced under our conditions, as assayed with an NO-sensitive electrode (World Precision Instruments, Sarasota, FL) (5). Hence, even if this NO\(^{-}\) reacts stoichiometrically with endogenous O\(_2^-\), only a low micromolar level of OONO\(^{-}\) is generated.

**Cortical Cell Culture, Drug Treatments, and Neurotoxicity Assays.** Cerebrocortical cultures were prepared from embryonic day 15 or 16 Sprague–Dawley rats as described (5, 15). Three-week-old cultures were exposed for 10 min to 2 mM or 300 \(\mu\)M NMDA/5 \(\mu\)M glycine in Mg\(^2+-\)-free Earle’s balanced salt solution (EBSS). After the 10-min exposure to NMDA, cultures were rinsed with EBSS, and their original medium was restored. For exposure to nitric oxide, superoxide anion, or peroxynitrite, the various reagents were added in concentrated form to the culture medium and very gently mixed. The cultures were then incubated for the times indicated in the figure. Neurotoxicity was determined by the failure to exclude trypan blue or the leakage of the enzyme lactate dehydrogenase (LDH), as described (5, 15).

**Propidium Iodide DNA Staining of Apoptotic Nuclei.** Cortical cells on glass coverslips were stained with propidium iodide (20 \(\mu\)g/ml, 5 min), as described (17). Cortical neurons were visualized by confocal laser scanning microscopy (Noran), and apoptotic nuclei were counted. The low percentage of cells with spontaneously apoptotic nuclei at time 0 in control cultures did not result in detectable LDH leakage.

**Analysis of DNA Fragmentation in Agarose Gels.** Cortical cells (1 \(\times\) 10\(^5\)) were lysed at 37°C for 60 min in a buffer containing 0.5% Triton X-100, 5 mM Tris buffer (pH 7.4), and 20 mM EDTA. The lysate was then incubated in proteinase K (100 \(\mu\)g/ml) for 2 h at 37°C. After RNase treatment for 1 h at 37°C, DNA was extracted with an equal volume of phenol/chloroform, 1:1, and precipitated with 1/10th vol 7 M ammonium acetate and 2.5 vol ice-cold ethanol at room temperature for 1 h. DNA samples were washed on 1.4% agarose gels and run at 90 V for ~2 h (18).

**In Situ Labeling of Nuclear DNA Fragments.** Cortical cell cultures were stained by the TUNEL technique (dUTP-mediated dUTP-biotin nick end-labeling), as described (19). Apoptotic cells could be discriminated morphologically by the presence of condensed brown nuclei in contrast to necrotic cells.

**Apoptotic Cell Death Detection by ELISA.** This assay is based on the quantitative sandwich-enzyme-immunooassay principle using mouse monoclonal antibodies directed against DNA and histones (Boehringer Mannheim kit, Cat. no. 1544 675). This ELISA provides a quantitative in vitro determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cortical cell lysates, as determined spectrophotometrically at 405 nm. The presence of mono- and oligonucleosomes is a feature of cells undergoing apoptosis (14, 18, 20).

**RESULTS AND DISCUSSION**

Assessment of Apoptosis and Necrosis After NMDS or Peroxynitrite. Excitotoxic damage was produced by adding NMDS to cortical cell cultures. For free radical-induced damage, we generated NO\(^{-}\) and O\(_2^-\), which react to form peroxynitrite (OONO\(^{-}\)) (4), using three separate methods to insure the validity of the results. SIN-1 generates both NO\(^{-}\) and O\(_2^-\), thereby producing OONO\(^{-}\) (16). SNOC generates NO\(^{-}\) that reacts with endogenous O\(_2^-\) in cortical cell cultures to produce OONO\(^{-}\) and consequent neuronal injury (5). We also synthesized peroxynitrite by stopped-flow reaction kinetics (5). All three methods yielded concordant results.

In this study, evidence for apoptotic-like features included the following: apoptotic nuclei by propidium iodide staining observed with confocal microscopy, the TUNEL technique as evidence for DNA damage, ELISA with anti-histone/DNA monoclonal antibodies as further evidence for DNA damage, and agarose gel electrophoresis to detect chromatin fragmentation. In contrast, lack of membrane integrity associated with necrosis was documented by the leakage of LDH or the failure to exclude trypan blue. Control experiments revealed that under our conditions neither NMDS nor OONO\(^{-}\) produced LDH leakage or DNA fragmentation in astrocyte cultures without neurons, indicating that it was the neurons that were damaged in the mixed neuronal/glial cortical cultures.

**Time Course of Neuronal Cell Death After Intense or Mild Excitotoxic- or Free Radical-Related Insults.** By the criterion of trypan blue exclusion, exposure to relatively mild insults (300 \(\mu\)M NMDS for 10 min or nominally 10 \(\mu\)M OONO\(^{-}\)) led to slower onset and less overall neuronal cell death than exposure to more intense insults (2 mM NMDS for 10 min or nominally 100 \(\mu\)M OONO\(^{-}\)) (Table 1). Under our conditions, intensive insults led to toxicity of nearly half of the neurons within 30 min and three-quarters of the neurons within 3 hr. In contrast, with more mild insults, toxicity did not affect three-quarters of the neurons until 18 hr (Table 1). Failure to exclude trypan blue reflects a loss of plasma membrane integrity associated with necrosis. Thus, lysis occurred earlier and in a greater percentage of cortical neurons exposed to more intense excitotoxic or free radical-related insults.

**Necrosis Quickly Follows Relatively Acute/Intensive Neuronal Insults.** A second assay verified these results, demonstrating leakage of LDH under similar conditions (Fig. 1). After a massive insult, acute release of LDH reflected cell lysis. Leakage of LDH occurred in <3 hr after relatively intense insults related to excitotoxins or free radicals (Fig. 1B and F), whereas it was evident only after 18 h in the face of more mild insults (Fig. 1A and E).

As early as 10 or 20 min after an intense insult with 100 \(\mu\)M OONO\(^{-}\), many neurons were swelled, and none of the neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30 min</th>
<th>6 h</th>
<th>18 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NMDS (300 (\mu)M)</td>
<td>16 ± 6.0</td>
<td>37 ± 7.0</td>
<td>84 ± 2.5</td>
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<td>NMDS (2 (\mu)M)</td>
<td>47 ± 2.5*</td>
<td>73 ± 5.5*</td>
<td>93 ± 2.0*</td>
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<tr>
<td>Peroxynitrite (10 (\mu)M)</td>
<td>21 ± 5.0</td>
<td>48 ± 4.0</td>
<td>70 ± 3.5</td>
</tr>
<tr>
<td>Peroxynitrite (100 (\mu)M)</td>
<td>45 ± 6.0§</td>
<td>84 ± 5.5§</td>
<td>100§</td>
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Values represent means ± SEMs, expressed as a percentage of neurons in the control cultures. Control cultures contained ~1000 neurons in 15 microscopic fields (each field scored at >200). Trypan blue uptake was measured at 30 min, 3 h, and 18 h after exposure. Statistical comparisons consisted of an ANOVA followed by Scheffé multiple comparison of means (\(t, P < 0.05\) and *; \(t, P < 0.01\) compared with 300 \(\mu\)M NMDS at the corresponding time points; \(\dagger, P < 0.05\) and \(\ddagger, P < 0.01\) compared with 10 \(\mu\)M OONO\(^{-}\) at the corresponding time points).
that time (Fig. 1B). During the necrotic process, neuronal cell bodies and nuclei swelled in contradistinction to the nuclear condensation evident during apoptosis. NMDA receptor antagonists D-2-amino-5-phosphono pentanoate (AP5, 200 μM–2 mM) and dizocilpine (MK-801, 1–10 μM) prevented both NMDA-induced necrosis and apoptosis in our system but were ineffective after exposure to free radicals or peroxynitrite (ref. 4 and data not shown).

To generate low levels of peroxynitrite (at most a few micromolar), we used 1 mM SIN-1 or 200 μM SNOC. Alternatively, we synthesized peroxynitrite and directly exposed the cortical cultures to low micromolar levels. Under our conditions, exposure to these relatively low concentrations of peroxynitrite resulted in delayed injury that was apoptotic in nature. For example, an increased number of apoptotic nuclei was observed 18 h after insult compared with 3 h (Figs. 1 C–E and 24 b–d). In these experiments, LDH levels eventually rose but only after many hours. This rise in LDH reflects the previously reported observation that apoptotic cells, under tissue culture conditions, will eventually leak their cytoplasmic contents because they do not undergo phagocytosis as in vivo. However, in the initial phases of apoptosis the integrity of the

![Figure 1](https://example.com/fig1.png)

**FIG. 1.** Time course of neurotoxicity induced by NMDA, SIN-1, SNOC, or peroxynitrite (OONO⁻). Dose and time dependence of NMDA (A, B) and OONO⁻ (E, F) on neuronal death, as determined by LDH leakage and number of apoptotic nuclei. Apoptotic nuclei increased most dramatically with lower concentrations of NMDA (300 μM) or OONO⁻ (10 μM). In contrast, LDH levels rose faster with higher concentrations of NMDA (2 mM) or OONO⁻ (100 μM). Time-dependent effect of SIN-1 (C) and SNOC (D) on neuronal death. Apoptotic nuclei and LDH levels increased significantly after 18 h compared with 3 h. Data shown are means ± SEMs (n = 9 cortical cultures). Apoptotic nuclei were counted in 15–20 fields in each culture at ×400 and expressed as a percentage of total neuronal nuclei. Statistical comparisons consisted of an ANOVA followed by a Scheffé multiple comparison of means (*, P < 0.01 compared to LDH levels at 3 h; †, P < 0.01 compared to the number of apoptotic nuclei after 3 h; **, P < 0.01 compared to LDH levels after 30 min).

![Figure 2](https://example.com/fig2.png)

**FIG. 2.** Confocal microscopy images of propidium iodide-stained neurons exposed to 200 μM SNOC (A) or 100 μM OONO⁻ (B). (A) (a–d) No apoptotic nuclei were observed in control neuronal cells (a) but were seen as highly fluorescent, condensed bodies after exposure to SNOC at 8 h (b), 12 h (c), and 18 h (d). (B) (a–d) No apoptotic nuclei were observed in the control neurons (a) or in neurons exposed to 100 μM OONO⁻ (b–d). Instead, exposure to 100 μM OONO⁻ for 30 min (b) or 2 h (c and d at a higher magnification) induced necrosis. Necrotic neurons were swollen, contained enlarged nuclei, and displayed the same intensity of fluorescence as control neurons. (Bars = 15 μm.)
plasma membrane is preserved, most functions of the membrane remain unchanged, and vital dyes such as trypan blue are excluded. Thus, an effect of the apoptotic process is to spare neighboring cells from exposure to the spillage of toxic products (21, 22).

In contrast, the loss of membrane structural integrity is an early event in necrosis (23). Thus the early appearance of LDH after intensive excitotoxic (Fig. 1B) or free radical-related (Fig. 1F) injury reflects necrosis. Consistent with this interpretation is the finding that after intensive insults, a substantial number of apoptotic nuclei did not develop at any time point, although the neurons swelled and died as evidenced by LDH leakage into the medium (Figs. 1 B and F and 2B b–d).

**Apoptosis with DNA Fragmentation.** Additional evidence was sought for apoptosis of neurons 12–18 h after exposure to the less intense insults (300 μM NMDA for 10 min, 1 mM SIN-1, 200 μM SNOC, or 10 μM peroxynitrite). In each case, genomic DNA from cortical cultures displayed a fragmentation pattern associated with apoptosis. As an example, Fig. 3 illustrates DNA fragmentation on an ethidium bromide-stained agarose gel produced 12 h after exposure to 200 μM SNOC (lane 2). At 2–3 h after exposure to 200 μM SNOC, nothing appeared on the gel. In contrast, 2 or 3 h after exposure to a severe insult (100 μM OONO⁻), agarose gel electrophoresis showed a smear of DNA due to random cleavage of base pairs during the necrotic process (Fig. 3, lanes 4 and 5). To prove that neurons were affected, DNA obtained from pure astrocyte cultures, rather than from mixed neuronal/glial cultures, did not manifest DNA fragmentation or smearing after these insults.

**Apoptotic Features Detected by TUNEL Technique and by ELISA for Histone-Associated DNA Fragments.** As mentioned above, rather than a single characteristic, there are several features that should be present to suggest the occurrence of apoptosis in neurons. For this reason, we performed two other techniques, TUNEL and ELISA for histone-associated DNA fragments (Figs. 4 and 5). With the TUNEL technique it is possible to discriminate morphologically the apoptotic nuclei by the presence of strand breaks in the DNA (evidenced by labeling the nicked ends of DNA) from areas of labeled necrotic cells, in which random DNA degradation occurs. However, any random DNA cleavage can eventually expose a 3' OH strand break that can potentially be labeled by the TUNEL technique. This may or may not be detected, but, in any event, it is the coexistence of TUNEL positivity with the aforementioned morphological changes that demonstrates apoptosis. The ELISA uses monoclonal antibodies to detect an increase in histone proteins that are associated with the fragmented DNA of the mono- and oligonucleosomes observed in the cytoplasm of apoptotic neurons.

Relatively intense insults with 2 mM NMDA or 100 μM OONO⁻ did not result in apoptotic nuclei detectable by TUNEL. On the other hand, 12 to 18 h after a relatively less intense insult (300 μM NMDA, 1 mM SIN-1, 200 μM SNOC, or 10 μM OONO⁻), a large number of apoptotic nuclei were clearly visible by TUNEL (e.g., Figs. 4 B and C for 1 mM SIN-1 at 18 h). Quantification of the number of nuclei observed by TUNEL revealed that 12 h after exposure to 200 μM SNOC, 40% of the neurons had apoptotic nuclei. A similar number was observed by propidium iodide staining visualized with confocal microscopy (data not shown). By 18 h after exposure to 200 μM SNOC,
hours after less severe insults with 300 μM NMDA or NO/O$_2^-$ donors generating ≤10 μM OONO$^-$, SOD/catalase could attenuate free radical-induced neurotoxicity of predominantly apoptotic nature, as long as peroxynitrite had not yet been formed. NMDA antagonists were effective in preventing necrotic or apoptotic neurotoxicity when coadministered with excitotoxin exposure but were ineffective in conjunction with free radical exposure because this step is presumably downstream from NMDA-receptor activation. Although the findings of this study occurred in vitro, both necrotic and apoptotic neuronal cell death have also been reported in vivo after excitotoxic/free radical insults. Therefore, if our findings hold true in vivo, they suggest that the intensity of the original insult may determine the ensuing pathway to either necrotic or apoptotic neuronal death. For example, acute neurologic insults—e.g., the ischemic core of a stroke—might be expected to result in necrosis, whereas chronic, less intense, or more prolonged insults may result in apoptosis. In vivo, apoptosis may also help avoid secondary injury to neighboring neurons, for instance in the penumbra of an ischemic stroke, because the cytoplasmic contents would not be spilled. The nature of the original insult as well as the decision to enter into the necrotic versus the apoptotic pathway might have therapeutic consequences in terms of the possible effectiveness of SOD/catalase or NMDA antagonists.

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