

Cytoskeletal Breakdown and Apoptosis Elicited by NO Donors in Cerebellar Granule Cells Require NMDA Receptor Activation

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Abstract: We have recently demonstrated that nitric oxide (NO) donors can trigger either apoptosis or necrosis of neurons as a function of the intensity of the exposure. Here, we show that the apoptosis induced by the NO donors S-nitrosocysteine (SNOC) or S-nitroso-N-acetylpenicillamine (SNAP) in cultured cerebellar granule cells (CGCs) depends on NMDA receptor (NMDA-R) activation leading to intracellular Ca^{2+} overload. Early dissolution of actin filaments followed by breakdown of microtubules and nuclear lamins preceded the appearance of typical apoptotic features. NO donors induced tyrosine nitration in neurons, in a small population of contaminating astrocytes, and in cultures of cerebellar astroglial cells. However, astrocytes neither displayed cytoskeletal alterations nor underwent apoptosis. Competitive and uncompetitive NMDA receptor antagonists, such as D-aminophosphonovaleric acid and MK-801, did not influence tyrosine nitration but prevented the accumulation of intracellular Ca^{2+} , cytoskeletal breakdown, and apoptosis induced by either SNOC or SNAP in CGCs. Taken together, these data strongly suggest that Ca^{2+} influx through NMDA-R-gated ion channels is a critical event in CGC apoptosis induced by NO donors. **Key Words:** Apoptosis—Calcium—Cytoskeleton—Neurodegeneration—Nitric oxide. *J. Neurochem.* **67**, 2484–2493 (1996).

Nitric oxide (NO) plays a major physiological role in the nervous system (Garthwaite and Boulton, 1995). However, inappropriate generation of this mediator—possibly in conjunction with reactive oxygen species—has been also implicated in neuropathology (Zhang and Snyder, 1995). Neurotoxicity of NO and related species has been attributed, at least in part, to DNA damage followed by activation of poly(ADP-ribose) synthase, with consequent NAD^+ and ATP depletion (Zhang et al., 1994). However, NO toxicity may also originate from direct protein modifications. The NO group, in its various redox forms, can inhibit

iron sulfur enzymes and some nonheme iron proteins and thereby contribute to a breakdown in cellular energy supplies and ribonucleotide synthesis (Gross and Wolin, 1995). Furthermore, peroxynitrite ($ONOO^-$) generated from NO^* and $O_2^{\cdot-}$ (Stamler et al., 1992) can react with tyrosine residues, causing alterations of protein phosphorylation or perturbation of protein tertiary structure (Beckman et al., 1994). Finally, protein thiol groups are targets of NO-related species, and S-nitrosylation modifies protein function (Lipton et al., 1993). The individual importance of damage to such targets may be dependent on the mode of exposure and on the cell type.

The understanding of NO pathophysiology is also complicated by its interaction with other second messengers and mediators. Especially important in neurons is the interaction between NO and glutamate signaling systems. On one hand, NO can induce the release of neurotransmitter molecules (Hanbauer et al., 1992), such as glutamate (O'Dell et al., 1991; Montague et al., 1994). On the other hand, glutamate-stimulated Ca^{2+} signals elicit NO production by a Ca^{2+} - and calmodulin-sensitive NO synthase (Garthwaite et al., 1988; Bredt and Snyder, 1989). Such endogenously produced NO mediates, at least in part, glutamate toxicity in rat cortical cultures (Dawson et al., 1991). The

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Abbreviations used: AM, acetoxymethyl ester; APV, D-aminophosphonovaleric acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CGC, cerebellar granule cell; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; NMDA-R, NMDA receptor; NO, nitric oxide; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; SNAP, S-nitroso-N-acetylpenicillamine; SNOC, S-nitrosocysteine; SOD, superoxide dismutase; TRITC, tetramethylrhodamine B isothiocyanate.

redox species of NO is also important in determining the biological effect. For example, NO[•] reacts with O₂^{•-} to produce ONOO⁻ and its associated neurotoxicity, whereas NO⁺ decreases NMDA-receptor (NMDA-R) channel activation, thereby attenuating Ca²⁺ overload elicited by glutamate (Lipton et al., 1993); vice versa, there may be NO redox species that increase the intracellular Ca²⁺ concentration ([Ca²⁺]_i) (Richter et al., 1994).

In our previous work, we have demonstrated that cortical neurons or cerebellar granule cells (CGCs) undergo apoptosis after mild insult delivered by NO donors or NMDA-R agonists (Ankarcrone et al., 1995; Bonfoco et al., 1995a). Here, we examine whether Ca²⁺ signals are involved in the apoptosis elicited by NO donors. We investigated the effect of agents that either lowered [Ca²⁺]_i or prevented Ca²⁺ entry through NMDA-R channels after exposure to NO donors. Furthermore, because cytoskeletal damage can promote apoptosis of CGCs (Bonfoco et al., 1995b), we decided to investigate whether cytoskeletal alterations were also part of NO-induced neuronal apoptosis and the possible role of Ca²⁺ in their development.

MATERIALS AND METHODS

Chemicals

N-Lauroylsarcosine, poly-L-lysine (mol wt, 300,000), L-cysteine, Hoechst 33258, phalloidin-fluorescein isothiocyanate (FITC), and mouse monoclonal antibodies against α -tubulin, β -tubulin, or glial fibrillary acidic protein (GFAP) were obtained from Sigma. Phalloidin-tetramethylrhodamine B isothiocyanate (TRITC), propidium iodide, SYTO-13, and the acetoxymethyl esters (AMs) of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), calcein, and fura 2 were purchased from Molecular Probes (Eugene, OR, U.S.A.). MK-801 and D-aminophosphonovaleric acid (APV) were from Research Biochemicals International (Natick, MA, U.S.A.). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Calbiochem (San Diego, CA, U.S.A.). *S*-Nitrosocysteine (SNO) was prepared as described previously (Lei et al., 1992).

Cell cultures and exposure

CGCs were prepared from 7-day-old Sprague-Dawley rats, as described by Schousboe et al. (1989a) with small modifications (Ankarcrone et al., 1995). Glial cells were prepared as described (Hansson and Rönnbäck, 1989) and cultured in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum. Culture purity (>95%) was assessed in each preparation using GFAP staining as a marker for glial cells.

NO donors were added from stock solutions directly to the culture medium. In the experiments examining the effect of cell density on cytotoxicity, CGCs were seeded in wells containing a coverslip. Before the exposure, coverslips were removed to leave in the wells a small but intact ring of CGCs. Alternatively, after replacing the culture medium with an analogous, serum-free medium, a fraction of cells was gently scraped off, the dishes were washed free of possible debris, and the remaining intact adherent patch of cells was

reincubated in the original culture medium. NMDA-R antagonists (1 μ M MK-801 or 10 μ M APV) or superoxide dismutase (SOD; 5 kU/ml) was added as 200 \times concentrated stock solutions 10 min before the treatment with the NO donors.

Tyrosine nitration

After exposure, CGCs adherent to glass coverslips were fixed with 80% (vol/vol) methanol for 15 min at room temperature, rinsed in phosphate-buffered saline (PBS), and treated with 3% (vol/vol) H₂O₂ for 5 min to remove endogenous peroxidase activity. Mouse monoclonal IgG (1:60 dilution) or rabbit polyclonal (1:80) antibodies directed against nitrotyrosine (a kind gift of Dr. J. S. Beckman, University of Birmingham, Birmingham, AL, U.S.A.) were then added to the coverslips, which were incubated for 1 h at 25°C followed by incubation with a biotinylated secondary antibody. A DAKO LSAB kit based on the peroxidase reaction was used as the detection system (Beckman et al., 1994). A positive reaction yields a red precipitate at the antigen site. Mayer's hematoxylin solution was used for counter staining.

Microscopy

Cells were visualized either using a conventional fluorescence microscope (Leica, DM-IRB) or a scanning confocal microscope (Bio-Rad MRC 600 or Leica TCS-4D). The TCS-4D microscope was equipped with a high-power argon laser (UV), a krypton/argon laser (visible), and three independent fluorescence detectors. Confocal images were calibrated digitally using as reference an object micrometer. The horizontal width of the white figure labels serves as the calibration bar.

[Ca²⁺]_i measurements

Fluorescence measurements of [Ca²⁺]_i in fura 2-loaded CGCs or glial cells were made using a SPEX Fluorolog-2 system as described previously (Bonfoco et al., 1995b). Changes in the ratio of fluorescence intensities, with excitation wavelengths of 340 and 380 nm, were measured at predetermined times after exposure to NO donors or other additions. The emission was set at 510 nm, and [Ca²⁺]_i was calculated according to the method of Grynkiewicz et al. (1985).

Formation of apoptotic nuclei

Apoptotic nuclei were detected by propidium iodide staining (Bonfoco et al., 1995b) and scored in 15–20 fields for each slide. Their number was expressed as a percentage of total neuronal cells visible in the fields. We also double-stained glial and neuronal cultures with 10 μ M ethidium bromide and 600 nM calcein-AM to visualize cells with intact plasma membranes (calcein positive) versus cells with broken membranes (ethidium bromide positive).

Analysis of DNA fragmentation on agarose gels

Formation of high-molecular-weight DNA fragments was detected by field inversion gel electrophoresis as described previously in detail (Ankarcrone et al., 1995). Calibration of DNA sizes was performed using sets of pulse markers: chromosomes from *Saccharomyces cerevisiae* (225–2,200 kb) and λ -DNA (48.5 kbp). Gels were stained with ethidium bromide, visualized using a UV light source (305 nm), and photographed with Polaroid 665 positive/negative film. Oligonucleosomal DNA fragmentation was detected after DNA extraction from lysates of CGCs (1 \times 10⁷), as described elsewhere (Ankarcrone et al., 1995). Agarose gel electro-

phoresis was performed with a constant voltage (90 V) for 3 h (Wyllie, 1980).

Immunocytochemistry for cytoskeletal proteins

After exposure, coverslips were rinsed in PBS and fixed (2.5% paraformaldehyde for actin, a sequence of 80% methanol and acetone for β -tubulin, and a sequence of 2.5% paraformaldehyde and 0.2% Triton X-100 for α -tubulin and GFAP). For β -tubulin, coverslips were incubated at 37°C for 30 min with a mouse monoclonal antibody used at a 1:200 dilution. Then coverslips were rinsed in PBS and incubated with a secondary antibody [anti-mouse IgG F(ab)₂ fragment, fluorescein-conjugated] for 30 min at 37°C. For F-actin staining, coverslips were incubated for 20 min at room temperature with 100 nM phalloidin conjugated with either FITC or Texas Red. Coverslips were then rinsed with PBS and mounted in HEPES-buffered saline/glycerol (1:1 vol/vol) on microscope slides. Lamin staining was performed after permeabilization with 100% methanol for 15 min using an antibody kindly provided by Dr. John Eriksson (Åbo Akademi, Finland). Blocking of nonspecific staining was done with PBS and 1% bovine serum albumin for 15 min before incubation with rabbit anti-lamin antibodies (1:60) for 30 min at room temperature. Coverslips were rinsed three times with PBS and then exposed for 30 min to secondary antibodies (Texas Red-conjugated goat anti-rabbit, 1:80 dilution). After rinsing with PBS, coverslips were exposed to the nuclear stain SYTO-13 (2 μ M) for 20 min at room temperature. Subsequently, they were rinsed with PBS and mounted on glass slides for microscopy.

Determination of glutamate concentrations

Glutamate concentrations in cell culture supernatants were measured using an enzymatic assay kit (Boehringer, Mannheim, Germany). The assay is based on the formation of NADH catalyzed by glutamate dehydrogenase. The NADH subsequently reduces a tetrazolium dye in a diaphorase-mediated reaction. The detection limit was 2 μ M glutamate, as determined experimentally.

RESULTS

NO donors induce apoptotic cell death in CGCs

SNOC (200 μ M) or SNAP (200 μ M) triggered CGC death with characteristic apoptotic features. Following exposure to either of the two NO donors, the number of cells displaying condensed, highly fluorescent nuclei increased significantly between 6 and 18 h and finally reached ~75% after 24 h (Fig. 1). Results with SNOC or SNAP were identical, whereas SNOC preincubated for 24 h to release NO before exposure had no effect. For convenience, only the results with SNOC are illustrated. To score the number of viable cells remaining on the dishes after treatment, we stained cultures with the combination of calcein-AM plus ethidium bromide. Typically, membrane permeability of CGCs treated with SNOC or SNAP increased at late time points. Nuclei of apoptotic cells appeared condensed and red compared with viable green cells after 18 h (Fig. 2a

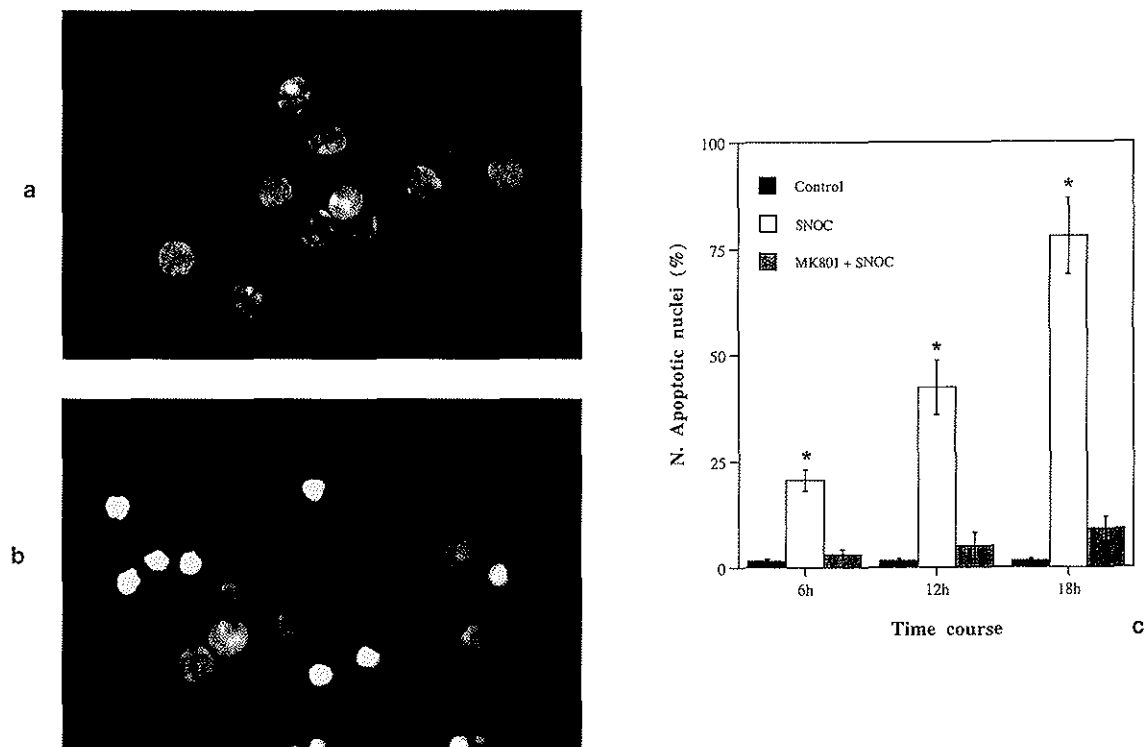


FIG. 1. Time course of formation of apoptotic nuclei in CGCs after exposure to 200 μ M SNOC. Cells in (a) untreated cultures and (b) cultures after a 12-h exposure were stained with propidium iodide; (c) apoptotic nuclei were counted in 10–15 fields in each culture and normalized to the total number of nuclei. MK801 + SNOC refers to preincubation with 1 μ M MK-801 for 5 min before exposure to SNOC. Apoptotic bodies were counted at the time points indicated. * $p < 0.01$ as determined by Student's *t* test.

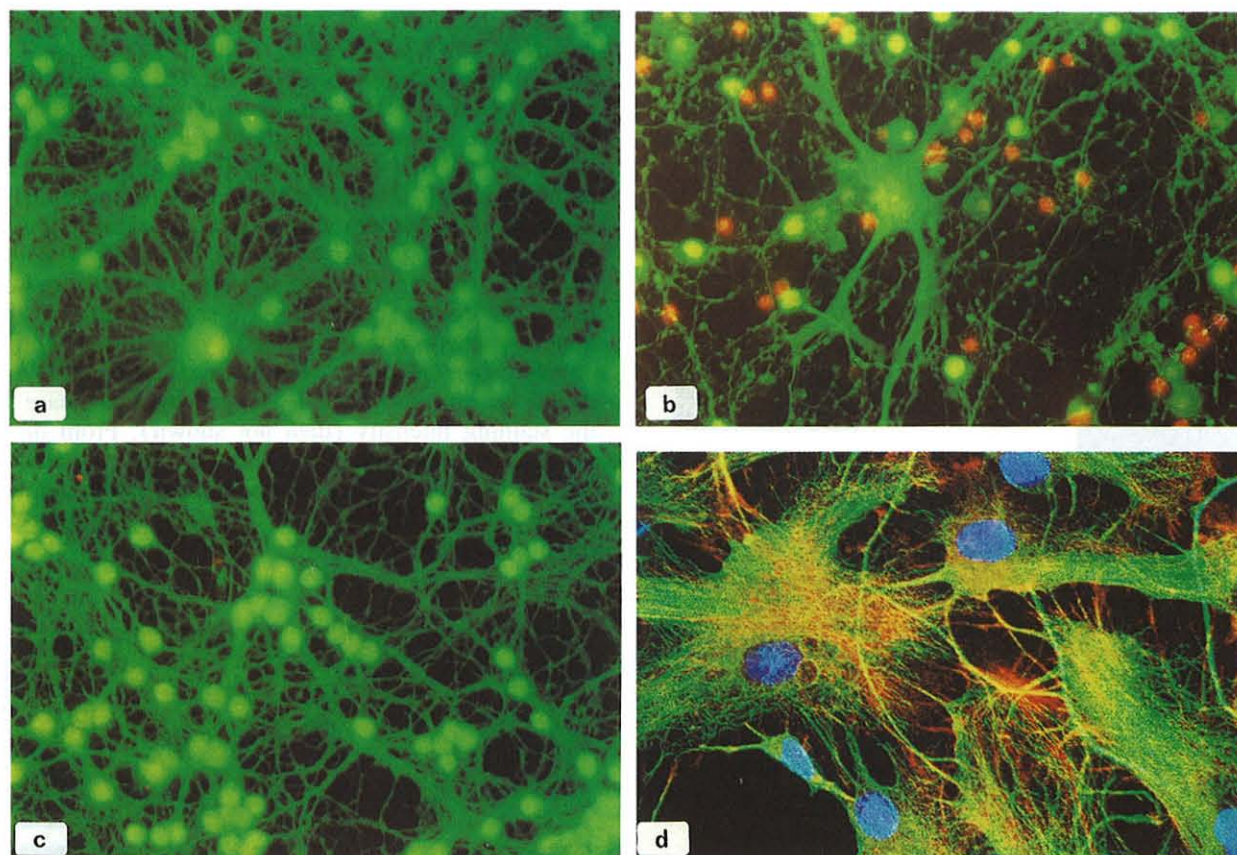


FIG. 2. SNOC induces death in neurons but not in astrocytes. CGC cultures were stained with calcein-AM plus ethidium bromide 18 h after treatment with (a) no challenge, (b) 200 μM SNOC, or (c) SNOC plus 1 μM MK-801. Cells with intact plasma membranes appear green (calcein retention), whereas cells with broken plasma membranes appear red and display pyknotic (and partially zeiotic) nuclei. Note in (b) that the single astrocyte present in the field was not affected by SNOC; (d) glial cultures were exposed to SNOC for 18 h and subsequently fixed. A triple stain was obtained using 1 $\mu\text{g}/\text{ml}$ Hoechst 33258 (blue nuclei), rhodamine-coupled phalloidin (red actin filaments), and anti- α -tubulin-FITC-conjugated monoclonal antibodies (green microtubule structures). Images were collected using a Leica TCS-4D confocal microscope. The white label serves as the calibration bar, which corresponds to a length of 18 μm for (a–c) and 11 μm for (d).

and b). In contrast, the few GFAP-positive cells present in our cultures, as well as pure astrocyte cultures treated with NO donors, showed neither necrosis nor apoptosis up to 5 days after the treatments (Fig. 2b and d). To establish whether endogenous NO generation would contribute to the NO donor toxicity, we preincubated cultures with *N*-monomethylarginine before the exposure to SNOC or SNAP. We found no difference in the extent of apoptosis in the cells treated with the inhibitor of NO synthase, in agreement with other findings in similar models (Demerle-Pallardy et al., 1991).

ONOO⁻ formed from NO^{*} and O₂^{*-} has been implicated as a major neurotoxic species. Here, the involvement of ONOO⁻ is suggested by the observation that pretreatment of CGCs with SOD reduced SNOC-induced apoptosis: After 18 h, untreated CGC cultures contained 7 \pm 2% apoptotic neurons, whereas treatment with SNOC caused apoptosis in 77 \pm 10% of the neuronal population, and cultures pretreated with SOD had only 40 \pm 6% apoptotic cells.

DNA fragmentation in CGCs exposed to NO donors

Progressive DNA cleavage to high- and low-molecular-weight fragments became evident 3 h after addition of NO donors. At the time the first apoptotic bodies were observed (6 h), 50-kbp fragments were also visible on gels. The results obtained with SNOC are illustrated in Fig. 3. Oligonucleosomal-sized (<20 kbp) DNA fragments were detected by conventional agarose gel electrophoresis beginning 12 h after exposure to NO donors (Fig. 3b).

Cytoskeletal alterations in CGC apoptosis induced by NO donors

Because CGCs exposed to cytoskeletal poisons undergo apoptosis with a time course similar to that observed in this study (Bonfoco et al., 1995b), we decided to investigate whether early cytoskeletal alterations were also a feature of the apoptotic program induced by the NO donors. We examined the early effects of SNAP or SNOC on actin filaments, microtu-

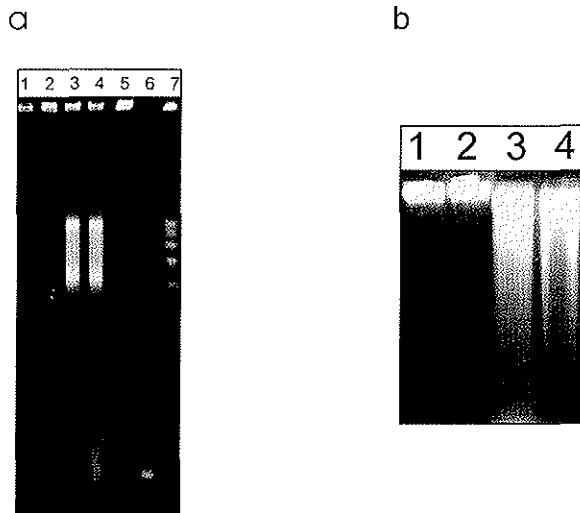


FIG. 3. Progressive formation of high-molecular-weight DNA fragments and DNA laddering in CGCs exposed to 200 μ M SNOC. Neurons were scraped off the plates and processed for DNA analysis by field-inversion gel electrophoresis (FIGE) and conventional agarose gel electrophoresis. **a:** FIGE gel. Lane 1, control; lane 2, 3 h after SNOC exposure; lane 3, 6 h after SNOC; lane 4, 12 h after SNOC; lane 5, 12 h after SNOC in CGCs pretreated with 1 μ M MK-801; lane 6, DNA molecular weight marker, 48 kbp; and lane 7, DNA molecular weight markers, 200–700 kbp. **b:** Conventional agarose gel electrophoresis. Lane 1, control; lane 2, 3 h after SNOC treatment; lane 3, after 12 h; lane 4, after 24 h.

bules, and lamins. Figure 4 illustrates the effect of SNOC on F-actin. As early as 1 h after exposure, actin filaments showed a lack of continuity (Fig. 4b). At 3 h after SNOC treatment, F-actin was clearly fragmented, and by 12 h the marked loss of actin fluorescence was indicative of a generalized depolymerization (Fig. 4c and d). F-actin dissolution was followed by microtubule fragmentation, which became conspicuous 6 h after addition of SNOC (Fig. 4f and g). Nuclear lamins also disintegrated after exposure to SNOC, coinciding with the formation of high-molecular-weight DNA fragments and the appearance of apoptotic bodies (Fig. 5). Phase-contrast microscopy revealed that the axonal structure was still intact when the microtubules and actin filaments had already fragmented. Thus, cytoskeletal changes involving actin filaments clearly preceded the appearance of distinct nuclear changes. Astrocytes did not display alterations in GFAP, microtubule, or actin filament staining (Figs. 2d and 4d).

Exposure to NO donors results in tyrosine nitration in both CGCs and astroglial cells

The experiments with SOD suggested that formation of ONOO⁻ was at least in part involved in the apoptosis elicited by NO donors. Nitration of tyrosines has been implicated in toxicity and cytoskeletal alteration induced by ONOO⁻ (Beckman et al., 1994).

Because apoptosis and cytoskeletal damage occurred extensively in CGCs treated with NO donors, we examined whether this would be reflected in a concomitant nitration of tyrosine residues. To detect nitrotyrosine in situ, we used either mono- or polyclonal antibodies in parallel incubations. Fifteen minutes after addition of SNAP or SNOC to CGC cultures, both neurons and astrocytes were positively stained regardless of the antibody used (Fig. 6a, b, and f). Staining persisted with similar intensity also in cells exposed to the NO donors for >1 h. As a negative control, the antibody reaction was quenched by addition of 1 mM nitrotyrosine (Fig. 6e). Pretreatment with SOD reduced tyrosine nitration, as judged by a decrease in the staining intensity (data not shown). From these findings, we conclude that although tyrosine nitration occurred in both neurons and astrocytes, additional factors influenced the initiation of apoptosis in the neuronal population.

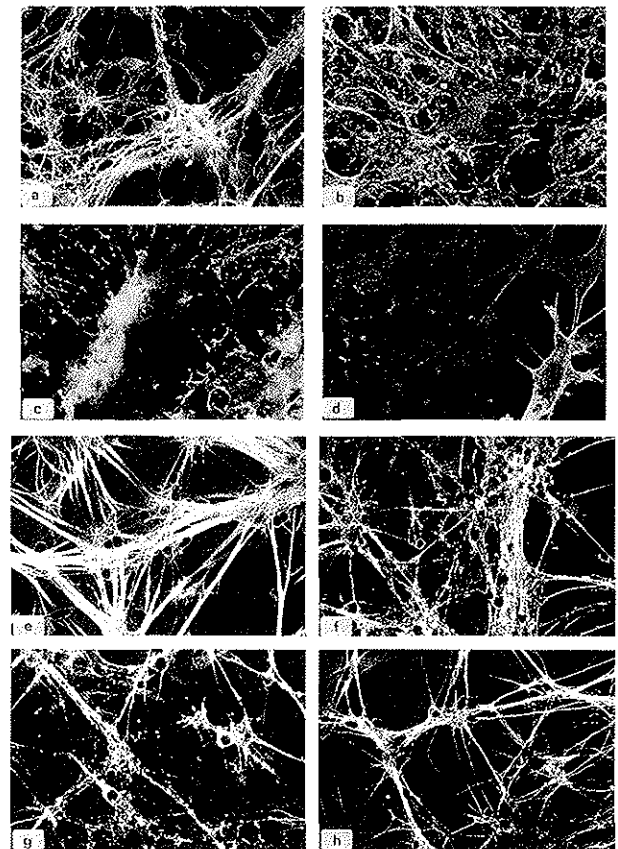


FIG. 4. F-actin and β -tubulin in CGCs exposed to 200 μ M SNOC. F-actin immunostaining was performed in neuronal cultures: control (a) and 1 (b), 6 (c), and 12 h (d) after SNOC treatment. Note in (d) the intact actin structure in the glial cell present in the field. β -Tubulin immunostaining was also done in control cultures (e) and 6 (f) and 12 h (g) after SNOC treatment; (h) β -tubulin 12 h after treatment with 1 μ M MK-801 and 200 μ M SNOC. The white label that serves as the calibration bar corresponds to a length of 16 (a–d) or 20 μ m (e–h) in the original sample.

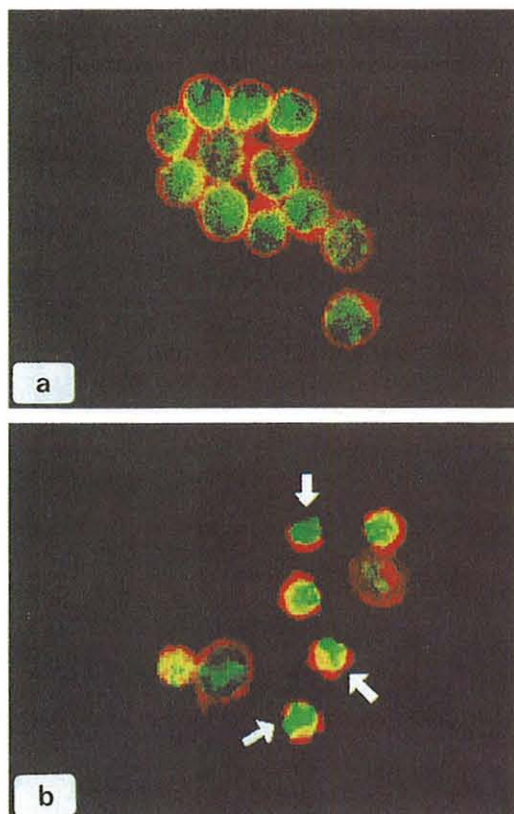


FIG. 5. Immunostaining of lamins in CGCs after NO treatment: (a) control and (b) 12 h after of 200 μM SNOC exposure. Lamins are stained red (TRITC-coupled secondary antibody). Nuclear counterstaining was performed using the green fluorescent dye SYTO-13. Arrows indicate lamin breakdown in condensed nuclei. The white label that serves as the calibration bar corresponds to a length of 10 μm in the original sample.

Treatment with SNOC increases $[\text{Ca}^{2+}]_i$ and alters Ca^{2+} responses to high K^+ levels

We decided to investigate whether other signals commonly eliciting neuronal apoptosis, i.e., Ca^{2+} overload, could be involved in this system. We initially measured $[\text{Ca}^{2+}]_i$ in individual neurons using fura 2-AM. Within 30 min of the addition of either of the two NO donors, the resting $[\text{Ca}^{2+}]_i$ increased from a control value of ~ 40 nM to an average of 90 nM. After 3 h in treated CGCs, $[\text{Ca}^{2+}]_i$ had increased further to reach a resting level of 120 nM (Fig. 7a). Treatment with the NO donors also caused alterations in stimulated Ca^{2+} responses. Exposure of untreated CGCs to high K^+ levels caused a rapid and pronounced increase in $[\text{Ca}^{2+}]_i$, whereas in neurons previously exposed to NO donors for 3 h, the same stimulus did not increase $[\text{Ca}^{2+}]_i$ further (Fig. 7). In contrast, resting $[\text{Ca}^{2+}]_i$ in glial cells (40 ± 6 nM) was unchanged after treatment with NO donors. To determine whether Ca^{2+} entry through neuronal membrane channels was involved, we selected either of two NMDA-R antagonists, MK-801 or APV. Either of these agents entirely prevented the increase in $[\text{Ca}^{2+}]_i$ induced by either

SNOC or SNAP and also restored the response to stimulation with high K^+ levels (Fig. 7b and c).

Prevention of cytoskeletal alterations and apoptotic cell death by MK-801, an NMDA-R or open-channel blocker

Next, we investigated whether restoring the resting $[\text{Ca}^{2+}]_i$ would also delay or prevent the toxicity of the NO donors. When CGCs were pretreated with MK-801, apoptosis was prevented even at late time points (Fig. 2). In addition, cytoskeletal structures remained intact (Fig. 4h), and DNA fragmentation as well as the late loss of membrane permeability did not occur (Figs. 3a and 1c). Because Mg^{2+} is known to inhibit NMDA-R activation, we tested whether raising Mg^{2+} concentrations in the culture medium would also protect from apoptosis induced by the NO donors. Indeed, increasing the Mg^{2+} concentration in the medium to 5 or 10 mM reduced the formation of apoptotic nuclei elicited by SNOC; 18 h after SNOC treatment there were $77 \pm 10\%$ apoptotic nuclei, whereas in the presence of 10 mM Mg^{2+} , the number of cells with apoptotic features decreased to $25 \pm 5\%$. Furthermore, when the intracellular Ca^{2+} buffering capacity was temporarily increased by loading cells with 10 μM BAPTA-AM before exposure to SNOC or SNAP, apoptotic cell death was also attenuated (8 h after SNOC treatment there were $31 \pm 4\%$ apoptotic nuclei in the absence and $15 \pm 3\%$ apoptotic nuclei in the presence of intracellular BAPTA).

NMDA-R activation can lead to the production of $\text{O}_2^{\cdot-}$ in cultures of CGCs or cortical neurons (Lafon-Cazal et al., 1993; Dugan et al., 1995). Thus, NMDA-R activation may have mediated the formation of ONOO^- and the consequent downstream events, i.e., tyrosine nitration. To investigate this possibility, we determined tyrosine nitration in CGCs treated with the NO donors in the presence of NMDA-R antagonists (either MK-801 or APV). As shown in Fig. 6c and d, these NMDA antagonists did not noticeably affect the nitration of tyrosine residues induced by SNOC.

Role of endogenous NMDA-R agonists in CGC apoptosis induced by NO donors

Finally, we investigated whether an endogenously released mediator or a component from the cell culture medium, such as glutamate, was responsible for the activation of NMDA-R channels in CGCs treated with NO donors. The mean glutamate concentration in the medium of CGCs exposed to NO donors remained consistently below 2 μM , i.e., a concentration that does not affect the viability of CGCs if added directly to the cell culture medium. Thus, the enhanced glutamate release, if any, that occurred during incubation with the NO donors was not sufficient to increase the overall glutamate concentration in the medium. Pretreatment of neurons with 1 mM pyruvate plus 35 U/ml glutamate pyruvate transaminase to degrade glutamate did not protect neurons from toxicity induced by NO donors. Finally, to examine further whether an increase

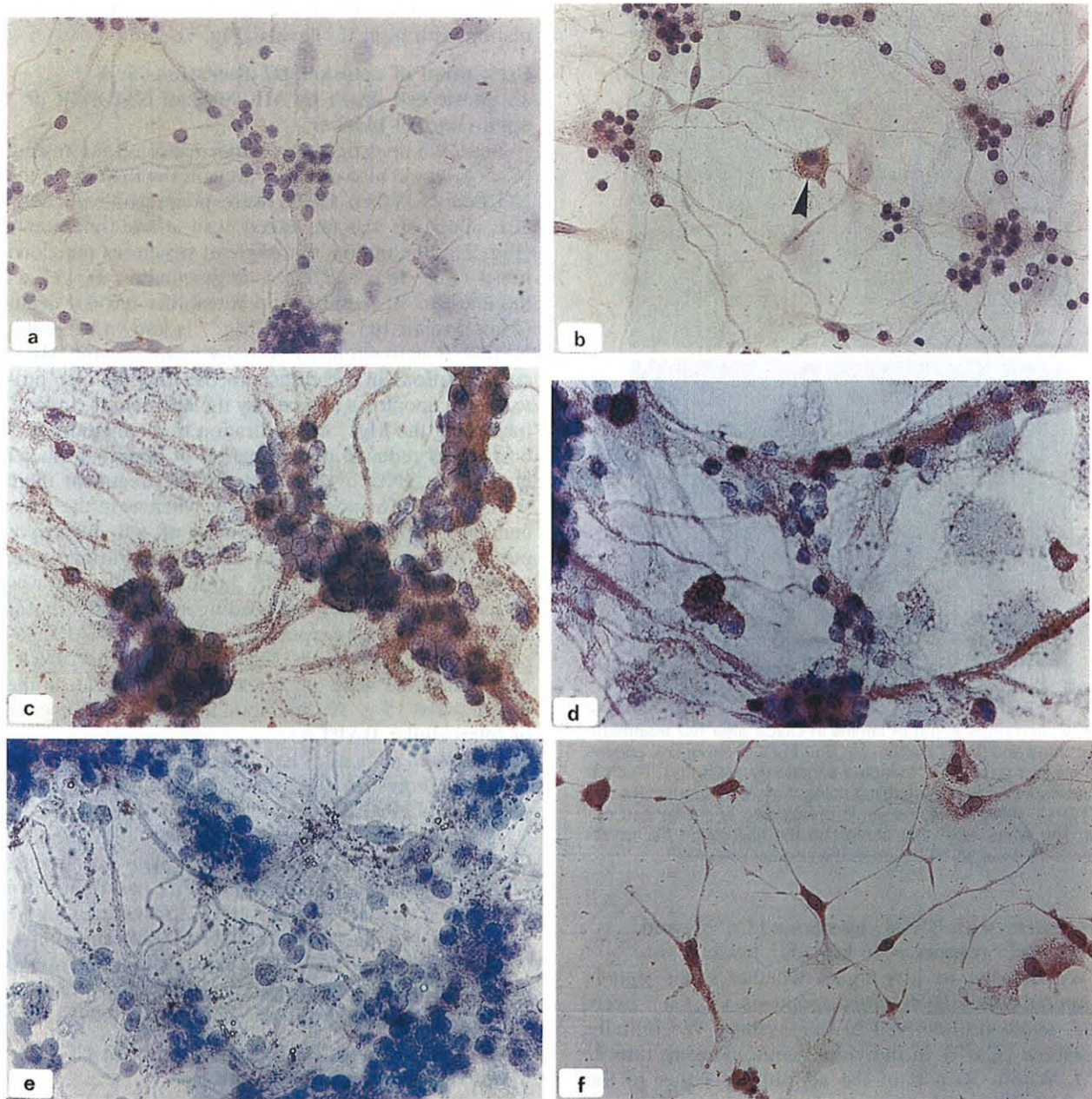
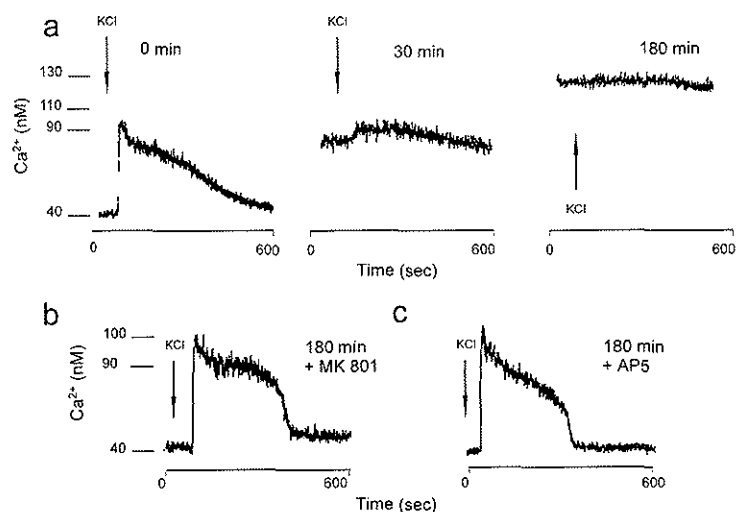


FIG. 6. Immunocytochemical detection of nitrotyrosine formation after treatment with SNOC. Cultures were fixed and stained with monoclonal antibodies directed against nitrotyrosine residues after exposure to $200 \mu\text{M}$ SNOC. The purple stain is due to the superimposition of red, specific for nitrotyrosine, and blue from the nuclear counterstain with Mayer's hematoxylin. **a:** Control neurons. **b:** CGCs and a single astrocyte (arrowhead) exposed to SNOC. **c:** Cultures pretreated with $1 \mu\text{M}$ MK-801 and then exposed to SNOC as in (b). **d:** As in (c) except that pretreatment was with $10 \mu\text{M}$ APV. **e:** Negative control shows competitive blocking of primary antibody with 1 mM free nitrotyrosine. **f:** Cerebellar astrocytes treated with SNOC. The white label used as the calibration bar corresponds to $23 \mu\text{m}$ (a, b, and f) or $15 \mu\text{m}$ (c–e).

in the overall concentration of any NMDA-R agonist in the culture medium was involved, we designed experiments in which neuronal cell densities were reduced to $\sim 10\%$ (see Materials and Methods for experimental details). We found that neither the time course and relative extent of apoptosis nor the sensitivity to MK-801 changed under these conditions.

Nevertheless, a local release of glutamate (or other NMDA-R agonists such as aspartate) may have produced a stimulation at protected synaptic sites. To test this hypothesis, we investigated whether APV, a glutamate antagonist that prevents the activation of the NMDA-R by inhibiting agonist binding, would block the toxic effects of SNOC or SNAP in CGCs. Indeed,

FIG. 7. $[Ca^{2+}]_i$ in CGCs after treatment with SNOC. **a:** CGCs exposed to 200 μM SNOC were loaded with 2 μM fura 2-AM 30 min before the time point indicated. Following depolarization with 50 mM KCl, $[Ca^{2+}]_i$ concentrations were recorded. The mean Ca^{2+} concentrations for four to six different neurons are displayed. Pretreatment of CGCs with **(b)** 1 μM MK-801 or **(c)** 10 μM APV (AP5) prevented the increase in resting $[Ca^{2+}]_i$ and restored a normal response to KCl depolarization 2 h after exposure to 200 μM SNOC.



pretreatment with APV prevented the increase in resting $[Ca^{2+}]_i$ and restored the response to high K^+ levels compromised by treatment with the NO donors (Fig. 7b and c). Also, cytoskeletal alterations, DNA fragmentation, and apoptosis elicited by SNOC or SNAP were prevented. Moreover, MK-801 or APV protected neurons from apoptosis when added up to 1 h after SNOC or SNAP (71 and 83% of the neuronal population were rescued by MK-801 and APV, respectively, as determined 18 h after SNOC treatment). Altogether, these results are consistent with the notion that the $ONOO^-$ (possibly together with other NO-related species) triggers the release of a glutamate-like agonist close to NMDA-R sites.

DISCUSSION

In our previous work, we have shown that mild insults with NO^* in conjunction with $O_2^{\cdot-}$ or NMDA-R agonists elicit apoptosis in cerebrocortical cultures and CGCs (Ankarcrona et al., 1995; Bonfoco et al., 1995a). Here, we went on to examine possible mechanisms by which NO donors can trigger neuronal apoptosis in the CGC model system. The advantage of these cultures is the homogeneity of the neuronal population, the low content of nonneuronal cells, and the absence of confounding factors such as the dependence of neuronal survival on growth factors and accessory cells.

Our results show that apoptosis elicited by NO donors in CGC cultures requires a Ca^{2+} signal triggered by activation of NMDA-R channels. The most likely explanation for these findings is that NO or a downstream species, e.g., $ONOO^-$ formed in the presence of $O_2^{\cdot-}$, stimulates the release of NMDA-R agonists, which subsequently act on NMDA-Rs. This is apparent from the findings that mechanistically different inhibitors of NMDA-Rs, MK-801 (Wong et al., 1986) and APV (Davies et al., 1981), prevented the increase in

the resting $[Ca^{2+}]_i$ and the onset of cytoskeletal alterations and apoptosis elicited by the NO donors. Further evidence for this supposition comes from the neuroprotective effects of Mg^{2+} in this culture system. Potential endogenous agonists include the classical amino acid neurotransmitters glutamate and aspartate (Watkins and Evans, 1981), which are both released from CGCs on depolarization (Levi et al., 1984; Schousboe et al., 1989b) or other low-molecular-weight substances, such as quinolinic acid. NO can stimulate neurotransmitter release (Hanbauer et al., 1992; Montague et al., 1994), and it can indirectly elicit neuronal depolarization by causing ATP depletion (Schousboe et al., 1989b). The depletion of ATP and depolarization would then lead to the release of excitatory amino acids. This mechanism of cellular depolarization after mitochondrial injury could result from NO^* or, more probably, $ONOO^-$ and is consistent with our prior findings concerning an initial mitochondrial dysfunction in neuronal apoptosis and necrosis elicited by excitotoxins (Ankarcrona et al., 1995).

Because the overall concentration of glutamate in the culture medium was below the level that can elicit neuronal death in this system and did not increase during the course of the experiments, it is likely that if glutamate was involved, it was released locally near NMDA-R sites. This assumption is also supported by the observation that the effects observed in CGCs exposed to NO donors were independent of the total number of neurons, i.e., even when the number of neurons was reduced to $\sim 10\%$, the relative extent of apoptosis and the sensitivity to NMDA-R antagonists were unchanged. Alternatively, the NO donors may have sensitized CGCs to the low amounts of NMDA-like agonists already present in the culture medium. The latter effect may have been the result of alterations of modulatory molecules, i.e., phosphorylating/dephosphorylating channel components. We are presently investigating these possibilities.

Although dysregulated calcium homeostasis is recognized as a central event in cell death (Choi, 1985; Nicotera et al., 1985, 1992, 1994; Hahn et al., 1988; Lipton and Rosenberg, 1994; Dubinsky, 1995), its causative role has been disputed (Harman and Maxwell, 1995). Prevention of apoptosis in our experimental system by an intracellular calcium chelator (BAPTA) and by blocking calcium influx (MK-801, APV, or high Mg^{2+} levels) further strengthens the evidence that increased $[Ca^{2+}]_i$ was not an epiphenomenon, but rather was directly involved in the apoptotic process.

Of the various species potentially generated by NO donors, ONOO⁻ can nitrate and modify proteins (by oxidation to disulfide or by nitrotyrosine formation) (Beckman et al., 1994) or stimulate lipid peroxidation (Radi et al., 1991). ONOO⁻ seems to be formed on exposure of CGC cultures to SNOC or SNAP, as judged immunocytochemically by the finding of extensive tyrosine nitration in situ. The partial protection by SOD supports the involvement of ONOO⁻ in CGC apoptosis. It is notable that the protective effect of the NMDA-R antagonists was apparently not due to a block in ONOO⁻ formation. The observation that MK-801 and APV failed to inhibit tyrosine nitration makes it unlikely that Ca^{2+} influx through NMDA-R channels was required to elicit the formation of ONOO⁻ from $O_2^{\cdot-}$ and NO donors. However, we do not exclude the possibility that $O_2^{\cdot-}$ production following Ca^{2+} entry through NMDA-Rs may have contributed to the cytotoxic effects of the NO donors.

Neither glial cells exposed to the NO donors nor CGCs in the presence of NMDA-R antagonists underwent apoptosis, despite tyrosine residues appearing heavily nitrated. These findings are consistent with the supposition that the apoptosis elicited by the NO donors in CGCs involves a specific event downstream to nitrotyrosine formation, which leads to subsequent NMDA-R stimulation. Alternatively, additional events different from tyrosine nitration, e.g., direct effects of other reactive species generated after exposure to the NO donors, may be required.

Cytoskeletal damage is a frequent component of neuronal cell death (Mattson, 1990; Lee et al., 1991). In particular, proteolytic disruption of cytoskeletal elements has been associated with the apoptotic program (Brancolini et al., 1995; Martin and Green, 1995; Kaylar et al., 1996). Modification and dissolution of F-actin are common features of both necrosis and apoptosis in several systems (Nicotera et al., 1992, 1994; Bonfoco et al., 1995a,b). In neuronal apoptosis, disruption of the microfilament network may be due to actin proteolysis mediated by ICE-like proteases (Brancolini et al., 1995; Kaylar et al., 1996). Our results suggest that disorganization of actin alone is not sufficient to produce the morphological changes associated with apoptosis in CGCs because cell shape and nuclear morphology apparently remained unaffected even when actin began to depolymerize. Nonetheless,

when microtubules and lamins were also significantly disrupted, cells displayed marked morphological changes, including nuclear pyknosis followed by changes in membrane permeability to ethidium bromide (occurring between 12 and 18 h). This is in agreement with our previous observations showing that primary microtubular damage can trigger CGC apoptosis (Bonfoco et al., 1995b).

In conclusion, we propose a novel mechanism to explain the neuronal demise elicited by NO donors such as SNOC or SNAP. This involves NO-mediated release of NMDA-R agonists with subsequent activation of NMDA-R channels and intracellular Ca^{2+} overload. The latter then activates downstream events leading to a sequential breakdown of major cytoskeletal proteins and apoptosis.

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