Peroxynitrite and Nitric Oxide Donors Induce Neuronal Apoptosis by Eliciting Autocrine Excitotoxicity

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Abstract

Endogenous generation of nitric oxide and its congeners, including peroxynitrite ($ONOO^-$), has been implicated in the mechanism of neuron loss in neurodegenerative diseases. Accordingly, nitric oxide donors and $ONOO^-$ can elicit both apoptosis and necrosis in neuron cultures. Here we show that nitric oxide donors and $ONOO^-$ are each able to trigger apoptosis of mouse cerebellar granule cells by an excitotoxic mechanism requiring exocytosis and NMDA receptor-mediated intracellular Ca^{2+} overload. This conclusion is supported by the following findings. Apoptosis was induced by various nitric oxide donors or by direct addition of $ONOO^-$ to differentiated cerebellar granule cell cultures that were sensitive to NMDA toxicity, but not in cerebellar granule cells that did not display NMDA-induced cell death (i.e. early days in culture) or in various glial cell populations. Donors of $ONOO^-$ or nitric oxide stimulated a sustained increase in intracellular Ca^{2+}, which was prevented by inhibitors of NMDA receptors, such as MK-801 and 5-phospho-aminovaleric acid, or by dampening neuronal electrical activity with high concentrations of extracellular Mg^{2+}. Moreover, these treatments and the exposure of cerebellar granule cells in nominally Ca^{2+}-free media prevented apoptotic cell death. Both the intracellular Ca^{2+} increase and apoptosis elicited by $ONOO^-$ or the nitric oxide donors were prevented by blocking exocytosis with tetanus toxin or botulinum neurotoxin C.

Introduction

Nitric oxide is a pleiotropic messenger in the central nervous system (Garthwaite and Boulton, 1995). It exerts physiological effects by regulating guanylate cyclase activity or possibly by mild, partially reversible, covalent protein modifications, such as *S*-nitrosylation (Stamler *et al.*, 1992a,b; Stamler, 1994). Under pathophysiological conditions (Crow and Beckman, 1995; Gross and Wolin, 1995) inappropriate production of nitric oxide may be involved in neurotoxicity (Dawson *et al.*, 1991; Zhang and Snyder, 1995). The targets and mechanisms of these adverse effects of nitric oxide are commonly thought to be different from those involved in its physiological actions. They may include DNA damage and irreversible protein modifications such as tyrosine nitration or thiol oxidation.

Enzymatically produced nitric oxide may undergo a large variety of non-enzymatic reactions e.g. with cellular thiols, metals or with superoxide ($O_2^{-\cdot}$). Products formed from these secondary reactions may have biological activities that differ considerably, which have been elucidated only in part (Lipton *et al.*, 1993; Stamler, 1994; Garthwaite and Boulton, 1995; Zhang and Snyder, 1995). In particular, peroxynitrite ($ONOO^-$), formed by the reaction of $NO^\cdot$ with $O_2^{-\cdot}$, has been implicated in cortical neuronal apoptosis (Bonfoco *et al.*, 1995; Dawson *et al.*, 1993), and as inducer of apoptosis in various cell lines (Estévez *et al.*, 1995; Lin *et al.*, 1995; Troy *et al.*, 1996). $ONOO^-$ can nitrate or hydroxylate protein tyrosine residues (Ischiropoulos *et al.*, 1992; Beckmann *et al.*, 1994; Crow and Beckman, 1995), it oxidizes thiols (Radi *et al.*, 1991; Stamler *et al.*, 1992a), and decomposes into $OH^\cdot$ and $NO_2^\cdot$ at neutral pH (Beckman *et al.*, 1990; Beckman *et al.*, 1995; Crow and Beckman, 1995). In neurons, the targets of $ONOO^-$ have been identified only partially. Possible cytotoxic mechanisms involve DNA damage and the activation of poly-(ADP-ribose) synthase with subsequent depletion of NAD and ATP (Zhang *et al.*, 1994), or mitochondrial damage (Bolanos *et al.*, 1995).

An entirely different paradigm of nitric oxide toxicity is based on its presynaptic actions. Nitric oxide is known to stimulate neurotransmitter release (O'Dell *et al.*, 1991; Hirsch *et al.*, 1993; Meffert *et al.*, 1994). Recently, it has been shown that nitric oxide stimulates exocytosis of neurotransmitters from synaptic vesicles probably by *S*-nitrosylating proteins responsible for the fusion of neurosecretory vesicles with the plasma membrane (Meffert *et al.*, 1996). In previous work we have also observed that NMDA receptor blockers prevent the toxicity of nitric oxide donors to cerebellar granule cells (CGC) (Bonfoco *et al.*, 1996). Therefore, it seems conceivable that nitric oxide elicits apoptosis by stimulating the exocytosis of excitotoxic neurotransmitters. This would imply that the mechanisms underlying the physiological and toxic actions of nitric oxide on neurons may involve, at least in part, similar pathways (e.g. neurotransmitter release by controlled *S*-nitrosylation of fusion proteins). In this case, the final outcome would be determined by the intensity of the insult

or by the metabolic condition of the cell. In several pathological situations, e.g. hypoxia (Rothman, 1984; Drejer *et al.*, 1985), hypoglycaemia (Wieloch, 1985; Sandberg *et al.*, 1986) and various forms of intoxication (Bagetta *et al.*, 1990; Turski *et al.*, 1991; Ishimaru *et al.*, 1992), unbalanced release of excitatory neurotransmitters seems to contribute significantly to toxicity, since NMDA receptor antagonists have proved to be neuroprotective. In line with such findings, we have shown (Bonfoco *et al.*, 1996) that inhibitors of NMDA receptors [MK-801 and D-2-amino-5-phosphonovaleric acid (APV)] prevent the characteristic series of events leading to the apoptosis of CGC after exposure to *S*-nitrosocysteine or *S*-nitroso-acetyl-penicillamine (SNAP). In the present study, we investigate the role of an autocrine excitotoxic mechanism in CGC apoptosis induced by nitric oxide donors or by direct exposure to $ONOO^-$.

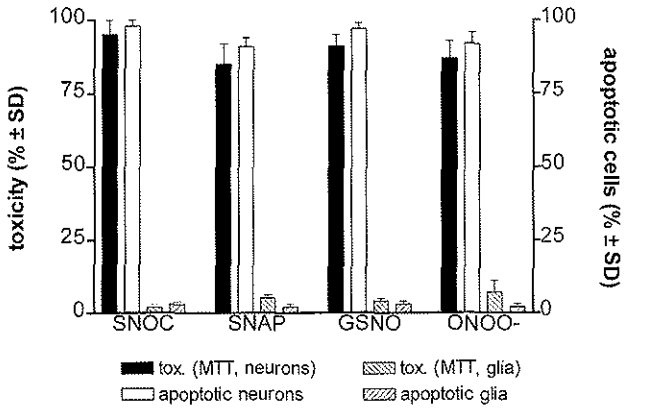


FIG. 1. Induction of CGC apoptosis by nitric oxide donors and $ONOO^-$. Cultures of CGC (9 DIV) were exposed to 50 μM *S*-nitrosocysteine (SNOC), SNAP, *S*-nitrosoglutathione (GSNO) or $ONOO^-$. Astroglial cultures were exposed to 150 μM of the nitric oxide donors or $ONOO^-$. Toxicity was determined after 16 h by counting apoptotic nuclei (H-33342 stain) or by the MTT assay. Data are mean ± SD of triplicate determinations.

Materials and methods

Materials

Cell culture dishes were from Greiner (Frickenhausen, Germany) and cell culture media from Life Technologies (Eggenstein, Germany) or Biochrom (Berlin, Germany). The acetoxymethyl-ester of fluo-3 (Fluo-3-AM), calcein-AM, SYTOX, SNAP and H-33342 were from Molecular Probes (Eugene, OR). Peroxynitrite (Beckman *et al.*, 1995), *S*-nitrosoglutathione (Hart, 1985) and *S*-nitrosocysteine (Lei *et al.*, 1992) were synthesized and quantitated as described. $ONOO^-$ (in 600 mM NaOH) and *S*-nitrosoglutathione (dried powder) were stored at −80°C under nitrogen for up to 6 weeks; *S*-nitrosocysteine was prepared freshly for each experiment. In control experiments, freshly prepared solutions were left to dissociate at neutral pH for 48 h, and then showed no significant toxicity. Dichlorokynurenate and 6,7-dichloroquinoxaline-2,3-dione were from Biomol (Hamburg, Germany). 1*H*[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one was from Alexis (Grünberg, Germany). Benzamide was from Aldrich (Steinheim, Germany), and *trans*-pyrrolidine dicarboxylic acid were from RBI (Cologne, Germany). *Clostridium botulinum* neurotoxin serotype C and tetanus toxin were purified as described (Schiavo and Montecucco, 1995). Solvents and inorganic salts were from Merck (Darmstadt, Germany) or Riedel-de Haen (Seelze, Germany). All other reagents not further specified were from Sigma.

Animals

Eight-day-old (for CGC) and 2-day-old (for glial cell cultures), specific pathogen-free BALB/c mice were obtained from the Animal Unit of the University of Konstanz. All experiments were performed in accordance with international guidelines to minimize pain and discomfort (US National Institutes of Health guidelines and European Community Council Directive 86/609/EEC)

Cell cultures

The CGC were prepared as described by Dreyer *et al.* (1985). Neurons were plated on dishes coated with 50 μg/ml poly-L-lysine (molecular weight >300 kDa) at a density of ~0.2 × 10^6 cells/cm^2 (640 000 cells/ml, 500 μl/well in 24-well plates) and cultured in Eagle's Basal Medium (BME, Gibco) supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM L-glutamine and penicillin–streptomycin. Forty-eight hours after plating, cytosine arabinoside (10 μM) was added to the cultures. Neurons were routinely used at 8–10 days *in vitro* (DIV) unless otherwise indicated. The frequency of glial fibrillary acid protein-positive cells was <5%. Cultures were usually exposed to nitric oxide donors in their own medium, or occasionally in controlled salt solution (CSS) (NaCl, 120 mM; HEPES, 25 mM; glucose, 15 mM; KCl, 30 mM; $CaCl_2$, 0–1.8 mM, Mg^{2+}, 0–0.5 mM; glycine, 0.01 mM) as indicated. *S*-nitrosocysteine was diluted for most experiments so that the working solution contained 0.05–0.25 mM HCl. For exposure to $ONOO^-$ we devised a protocol involving addition of small amounts of acid (HCl) sufficient to prevent alkalinization from the $ONOO^-$ stock solution. This procedure prevents putative receptor desensitization due to transient alkalinization. Solvents alone did not affect neuron survival even after continued culture for 6 days. Inhibitors were added routinely 10 min before exposure to nitric oxide donors. Glial cells were prepared as described (Hansson and Rönnbäck, 1989) and cultured in BME supplemented with 10% heat inactivated fetal calf serum. Cerebellar oligodendrocytes and primary murine microglia were kindly provided by Dr M. Jung (Konstanz) and Dr C. Nolte (Berlin), respectively.

In some experiments, polylysine coating was applied to a small area (~1 mm^2) within the culture well. Thirty minutes after plating, non-adherent cells were washed away to leave only a small patch of neurons (500–1000) on the polylysine spot. Although the total number of neurons within the dish was ≤0.5% of the normal number, the density of cells within the patch and the formation of the axonal/dendritic network after 8 DIV was similar to that in normally coated culture wells.

Microscopy

Cells stained with different fluorescent probes were imaged in a Leica DM-IRB microscope equipped with a video camera or connected to a TCS-4D UV/VIS confocal scanning system.

Measurement of $[Ca^{2+}]_i$

Dynamic changes of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured by imaging individual neurons loaded with a fluorescent Ca^{2+} indicator. Cells grown on plastic dishes were loaded in their original medium with 1 μM Fluo-3-AM for 15 min at 37°C. Then the medium was exchanged for CSS (with 0.5 mM Mg^{2+}, 1.8 mM $CaCl_2$, 30 mM KCl), and CGC were incubated for 10 min to allow complete de-esterification of fluo-3. The medium was then replaced with the original neuron-conditioned complete BME medium supplemented with 20 mM HEPES. The CGC were allowed to

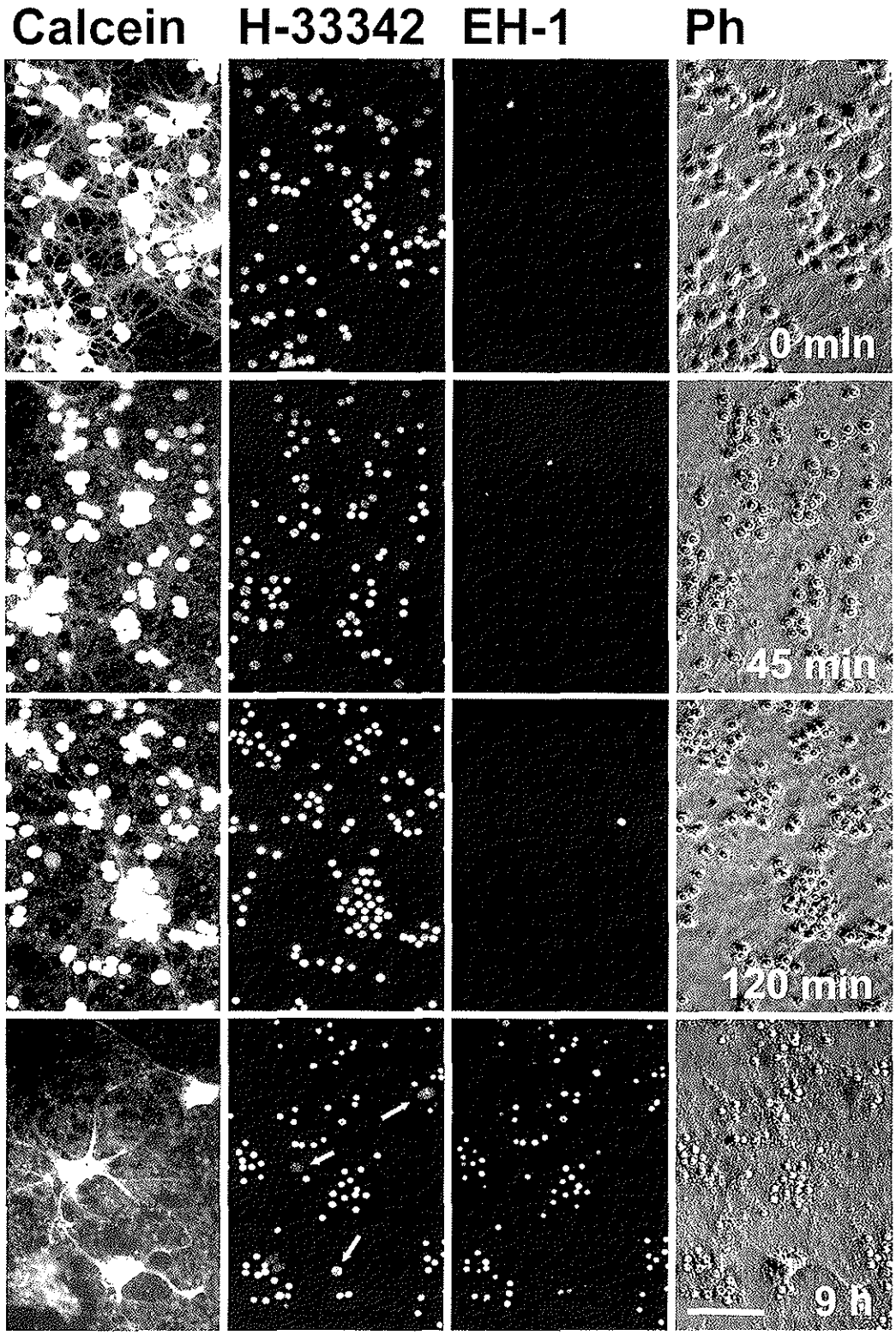


FIG. 2. Kinetics of cellular changes induced by exposure of CGC to $ONOO^-$. Parallel CGC cultures were exposed to 50 μM $ONOO^-$ and stained with three fluorescent markers 5 min before observation. At each time point a group of neurons was imaged sequentially under phase-contrast optics (Ph) and with three fluorescent filter sets (red fluorescence, EH-1; blue fluorescence, H-33342; green fluorescence, calcein-AM). After 45 min 50% of the neuronal bodies were clearly condensed and hyperfluorescent. After 120 min all neuronal nuclei were condensed but still excluded EH-1, and virtually all neurons accumulated calcein. After 9 h, neuronal membranes were broken but the nuclei did not detach from the culture dishes. Notably, the glial cells (arrows) in the field were not damaged (calcein retention, no condensation or EH-1 staining of their nuclei). Scale bar, 50 μm.

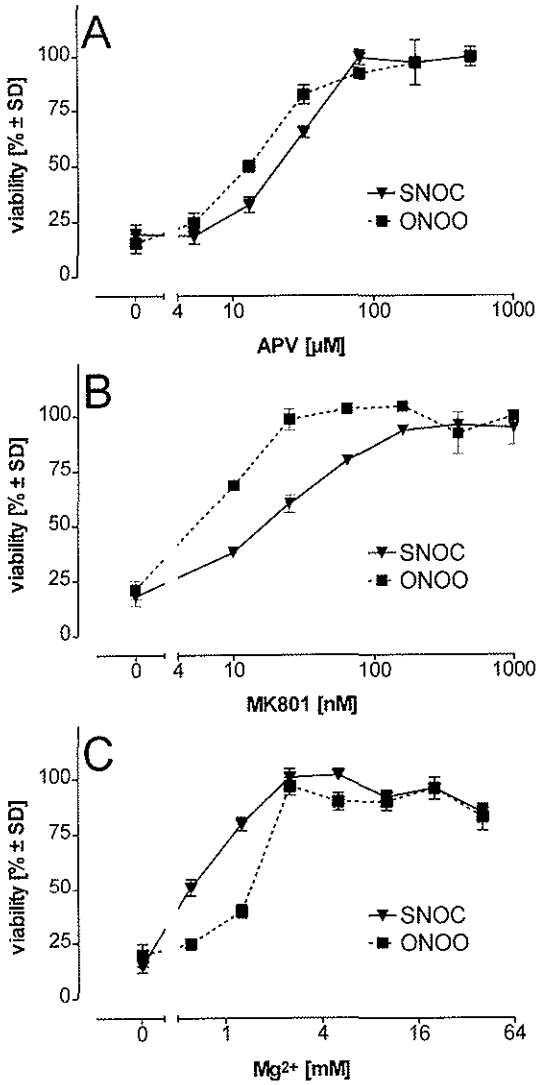


FIG. 3. Prevention of nitric oxide/$ONOO^-$ toxicity by NMDA receptor antagonists. Cerebellar granule cells were incubated with inhibitors for 30 min, before *S*-nitrosocysteine (SNOC; 10 μM) or $ONOO^-$ (50 μM) was added. Viability was determined after 16 h by the MTT assay. Data are mean ± SD of triplicate determinations.

equilibrate at room temperature for 10 min before exposure to $ONOO^-$ or to nitric oxide donors. Images were collected using 488 nm excitation and 520 nm emission wavelengths with a Leica DM-IRB microscope equipped with a 40× lens at intervals of 10 s (for nitric oxide donors) or 2 s (for chemical depolarization, i.e. addition of 50 mM KCl). The integration period was 100 ms per image with a Dage-72 (Dage-MTI, Michigan City, IN) CCD camera [756 (horizontal) × 581 (vertical) pixels]. Data were analysed using software from Imaging Corporation (St Catherines, Ontario, Canada). Relative mean fluorescence levels from defined areas corresponding to the position of neuronal cell bodies were recorded over 30 min and further processed using Excel 5.0 software (Microsoft). The mean fluorescence level of each marked cell was arbitrarily set to 1 at the beginning of each experiment. After each experiment, the increase in $[Ca^{2+}]_i$ triggered by K^+ depolarization (50 mM K^+) was measured in order to determine the reactivity of the neurons and the validity of the experimental conditions.

Viability assays

To determine cell membrane integrity and nuclear changes, CGC were loaded with 1 mM calcein-AM for 2 min (cells with intact membranes displayed green fluorescence) in the presence of 2 μM ethidium homodimer-1 (EH-1) (cells with broken membranes exhibit nuclear red fluorescence) and 500 ng/ml of the bisbenzimide dye H-33342 (cell-permeant, blue fluorescence). Alternatively, apoptosis and secondary lysis were quantitated by double-staining neuron cultures with 1 μg/ml H-33342 and 1 μM SYTOX (non-cell-permeant, green fluorescence). Apoptotic cells were characterized by condensed, highly fluorescent nuclei. In addition, the percentage of viable cells was quantitated by their capacity to reduce 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrasodium bromide (MTT) after incubation with 0.5 mg/ml MTT for 60 min (Mosmann, 1983). Reduction of MTT was usually measured 12–16 h after the treatments. For time-lapse experiments HEPES (pH 7.3, final concentration 20 mM) was added to the original culture medium. Cells were loaded for 15 min with 250 ng/ml Hoechst-33342 or used directly for phase-contrast microscopy. Control wells were always run on the same 24-well culture dish and scored after the end of the experiment. To quickly test membrane integrity at the end of these experiments, 90 μl trypan blue (0.4%) was added to cultures, which were bathed in 500 μl of medium and left to incubate for 5 min. After very gentle washing, trypan blue-positive cells were scored.

Statistics

Individual experiments were run in triplicate. Traces of fluo-3 fluorescence represented individual neurons, with 20–50 traces displayed per figure. Each of these experiments was repeated with at least three neuron preparations. Statistical significances were calculated on the original data sets using Student's *t*-test. When variances within the compared groups were not homogeneous we used the Welch test.

Results

Induction of cerebellar granule cells apoptosis by nitric oxide donors or $ONOO^-$

Exposure to various nitric oxide donors, i.e. *S*-nitrosocysteine, SNAP or S-nitrosoglutathione, resulted in CGC apoptosis (Fig. 1) with the typical features of nuclear pyknosis and chromatin condensation. Loss of MTT metabolizing activity (Fig. 1) as well as secondary membrane lysis (determined by the number of EH-1-positive nuclei) followed (Fig. 2). Mouse astroglial cultures were not affected by the same or three-fold higher concentrations of the nitric oxide donors (Fig. 1), even if exposure was prolonged for 48 h. Also, nitric oxide donors were not toxic for a mouse cerebellar oligodendrocyte line or primary murine microglia cultures (data not shown).

$ONOO^-$ is generally considered as the terminal neurotoxic species formed from $NO^\cdot$ plus $O_2^{-\cdot}$. Direct addition of this metabolite to CGC also elicited neuron death (Fig. 1). To further characterize the development of apoptosis induced by $ONOO^-$ or the nitric oxide donors we used time-lapse video microscopy (Fig. 2). Within 45–150 min after exposure to $ONOO^-$ or *S*-nitrosocysteine, 85–100% of all neuron nuclei condensed while the cell membrane remained intact. At the end of the experiment, i.e. after 150 min, the large majority (≥90%) of neurons with condensed nuclei excluded trypan blue or EH-1, and were able to accumulate calcein-AM. Membrane integrity was lost only several hours later, as determined in parallel cultures. Notably, apoptotic nuclei did not disintegrate and remained adherent to the culture plate together with the remnants of the neurons for >24 h (not shown). Virtually identical results were obtained with

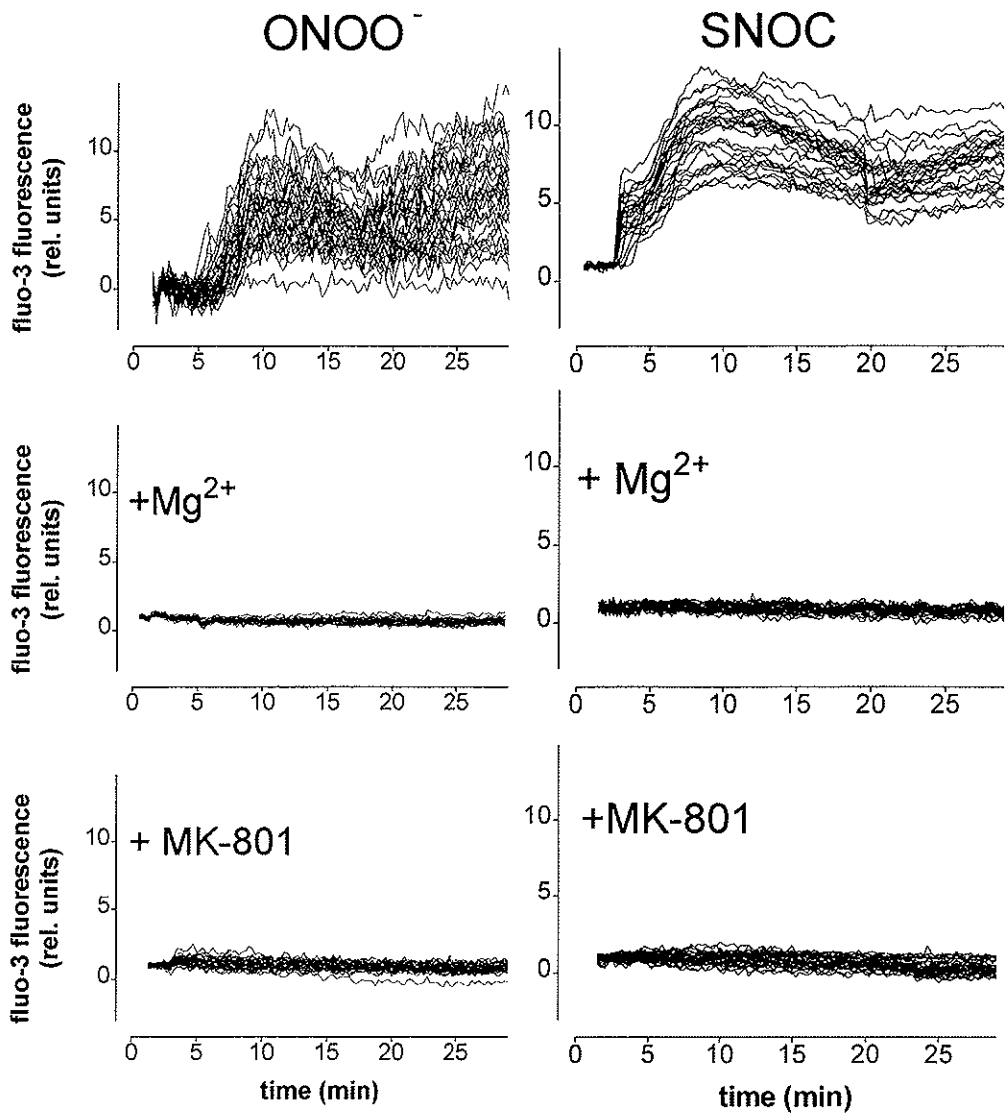


FIG. 4. Prevention of nitric oxide/$ONOO^-$-elicited increases in $[Ca^{2+}]_i$ by NMDA receptor inhibition. Cerebellar granule cells were loaded with fluo-3-AM for video imaging of $[Ca^{2+}]_i$. They were challenged with 88 μM $ONOO^-$ or 50 μM *S*-nitrosocysteine (SNOC). MK-801 (2 μM) or Mg^{2+} (10 mM) was added 10 min before the stimuli. Each trace represents an individual cell body. Each set of data is from two or three experiments. All data presented in the figure are from the same cell preparation.

$ONOO^-$ and *S*-nitrosocysteine. Figure 2 illustrates typical changes observed with $ONOO^-$.

The role of NMDA receptor in $ONOO^-$- and nitric oxide-induced apoptosis

We have previously observed that NMDA receptor antagonists protect rat CGC from *S*-nitrosocysteine-toxicity (Bonfoco *et al.*, 1996). Here we examined whether such intervention would also prevent the toxicity of the putatively distal and direct mediator of toxicity, $ONOO^-$. A competitive (APV) as well as a non-competitive (MK-801) inhibitor of the NMDA receptor prevented neurotoxicity of $ONOO^-$ (Fig. 3). Protection was complete, as pretreatment with MK-801 allowed the survival of $ONOO^-$-challenged neurons for at least 48 h. To further examine whether excitotoxicity was the pivotal mechanism leading to $ONOO^-$/nitric oxide toxicity, we increased the Mg^{2+} concentration in the medium. High $[Mg^{2+}]$ dampens neuron activity and blocks the opening of NMDA receptor-gated Ca^{2+} channels (Monyer *et al.*, 1992b; Rothman, 1983). This condition prevented the apoptosis induced by *S*-nitrosocysteine or $ONOO^-$ (Fig. 4). In addition, we used NMDA receptor inhibitors acting at the glycine costimulatory binding site (25 μM 5,7-dichlorokynurenate or 80 μM 6,7-dichloroquinoxaline-2,3-dione). Treatment with these agents reduced the toxicity of 50 μM $ONOO^-$ and *S*-nitrosocysteine by 45 and 55% respectively. The effective protection afforded by using competitive (APV) and non-competitive NMDA blockers and the similarity of the concentration response curves obtained with the inhibitors in *S*-nitrosocysteine or $ONOO^-$ treated neurons strongly suggest that these two agents acted by a common mechanism, e.g. by releasing an excitotoxic mediator that activates the NMDA receptor.

$ONOO^-$ and S-nitrosocysteine stimulate an increase in $[Ca^{2+}]_i$ in cerebellar granule cells via the NMDA receptor

To test whether $ONOO^-$ or the nitric oxide donors would elicit NMDA receptor-mediated Ca^{2+} influx, we measured $[Ca^{2+}]_i$ in CGC

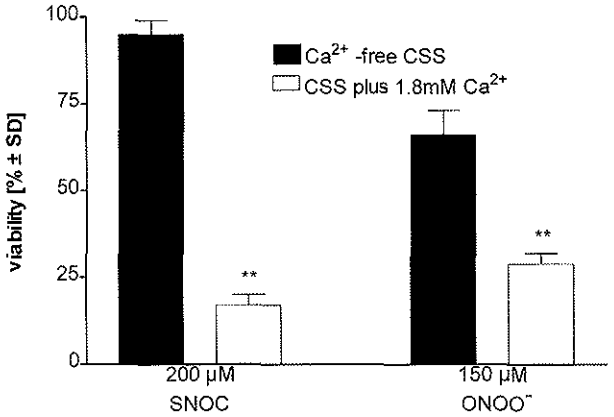


FIG. 5. Modulation of *S*-nitrosocysteine/$ONOO^-$ toxicity by the extracellular Ca^{2+} concentration. CGC were exposed to *S*-nitrosocysteine (SNOC) or $ONOO^-$ for 3 min before the original medium was rapidly removed and replaced by CSS without added Ca^{2+} (Ca^{2+}-free CSS) or by CSS with 1.8 mM added Ca^{2+}. Viability was determined after 16 h by MTT assay. Data are mean ± SD from triplicate determinations. $^{**}P < 0.01$, Ca^{2+}-free versus 1.8 mM Ca^{2+}.

immediately after the addition of *S*-nitrosocysteine or $ONOO^-$. Addition of $ONOO^-$ or *S*-nitrosocysteine elicited a rapid and sustained increase in $[Ca^{2+}]_i$ that was entirely abolished by pre-treatment with high Mg^{2+}, MK-801 (Fig. 4) or 500 µM APV (not shown). Then, to determine whether Ca^{2+} influx was causally involved in the toxicity of nitric oxide, we assessed the toxicity of *S*-nitrosocysteine in CSS in the presence or absence of added Ca^{2+} (1.8 mM). When exposure to *S*-nitrosocysteine was performed in nominally Ca^{2+}-free conditions, toxicity was virtually abolished (70–95% protection). Interestingly, when extracellular Ca^{2+} was removed after the addition of *S*-nitrosocysteine, toxicity was also reduced. A similar effect was observed with $ONOO^-$ (Fig. 5). In these experiments the original BME medium was replaced by CSS with or without 1.8 mM Ca^{2+}, 3 min after the addition of $ONOO^-$ (i.e. when most $ONOO^-$ was presumably dissociated). In line with these findings, the addition of 2 µM MK-801 2 min after treatment with *S*-nitrosocysteine or $ONOO^-$ rescued 81 ± 4 and 46 ± 5% of the neurons respectively.

Dependence of $ONOO^-$/nitric oxide-induced apoptosis on in vitro differentiation of cerebellar granule cells

Cerebellar granule cells change functional properties, receptor expression and consequently sensitivity towards excitotoxic stimuli during maturation. After plating and for the first few days *in vitro*, CGC are not sensitive to NMDA toxicity (Frandsen and Schousboe, 1990; Piani *et al.*, 1992; Rossi and Slater, 1993; Xia *et al.*, 1995). Therefore we examined the toxicity of *S*-nitrosocysteine and $ONOO^-$ during *in vitro* differentiation of CGC. The lethal effects of NMDA, *S*-nitrosocysteine and $ONOO^-$ were strongly dependent on the developmental stage (Fig. 6). This was not due to general lability of the neurons caused by energy deprivation, since supplementation of the culture medium with 20 mM glucose did not affect the toxicity of the nitric oxide donors, although it prolonged long-term survival of untreated cultures.

Similar sensitivity of DIV 5 and DIV 8 cultures to 200 µM $ONOO^-$ but a large difference at lower concentrations suggests that qualitatively different pathways of nitric oxide toxicity exist in CGC. However, the finding that MK801 (2 µM) and APV (500 µM) are also effective (100% protection) at concentrations up to 200 µM *S*-nitrosocysteine/$ONOO^-$/S-nitrosoglutathione suggests only a quantitative difference between the different cultures with respect to nitric oxide toxicity, i.e. DIV 5 cultures containing fewer synapses may require higher amounts of nitric oxide to release the same amount of neurotransmitter.

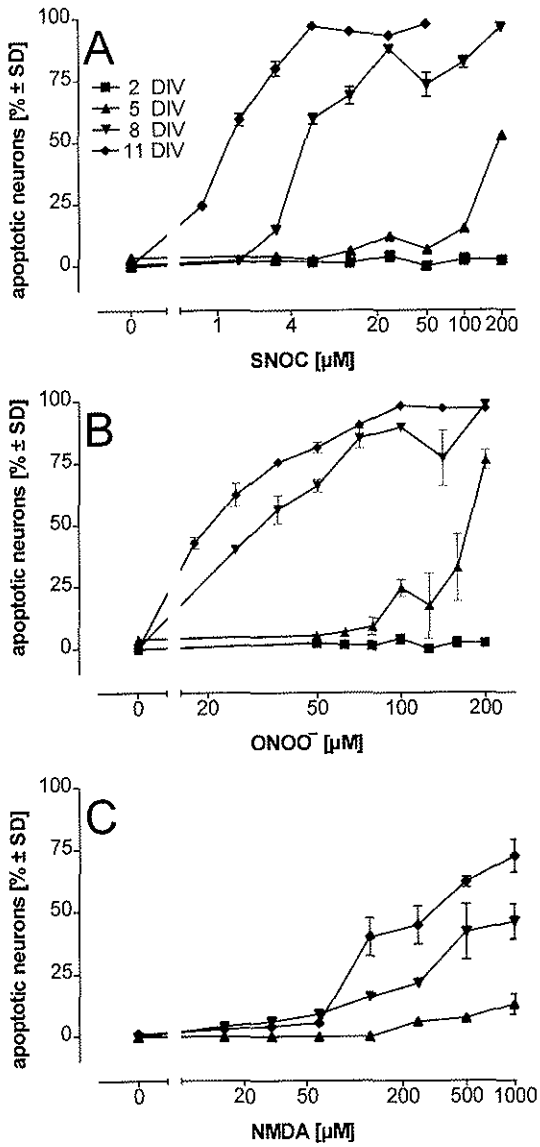


FIG. 6. Dependence of nitric oxide/$ONOO^-$-toxicity on the differentiation state of CGC. The CGC were used at 2, 5, 8 or 11 DIV. They were exposed to the indicated toxin concentrations for 24 h before they were fixed, stained with H-33342 and scored for the percentage of apoptotic neurons. Parallel toxicity determinations with the MTT method yielded essentially similar results. Data are mean ± SD of triplicate determinations. SNOC, *S*-nitrosocysteine.

Pharmacological characterization of nitric oxide-induced cerebellar granule cell apoptosis

The sensitivity of nitric oxide-induced apoptosis to pharmacological agents known to affect other actions of nitric oxide was also tested. These experiments suggested that the toxicity of exogenous nitric oxide or $ONOO^-$ was dependent neither on *de novo* protein synthesis nor on guanylate cyclase activation (Garthwaite *et al.*, 1995). Also, inhibitors of poly-(ADP-ribose) synthase were ineffective in this system (Table 1). In contrast, a protective effect was observed

using an enzymatic system to remove glutamate (glutamate–pyruvate transaminase plus pyruvate). Thus, in accordance with previous reports on the activating action of nitric oxide on the vesicle fusion machinery (Meffert *et al.*, 1994, 1996), $ONOO^-$ and nitric oxide donors may directly stimulate the release of glutamate from synaptic terminals.

Endogenous mediator release and autocrine toxicity of nitric oxide and $ONOO^-$ in cerebellar granule cells

To directly address the possibility that exocytosis of excitotoxic mediators is necessary for apoptosis induced by *S*-nitrosocysteine or $ONOO^-$, CGC were treated with clostridial toxins which are known to impair exocytosis by cleaving vesicle-docking proteins. Pretreatment for 2–10 h with either tetanus toxin or botulinum neurotoxin C markedly reduced the neurotoxicities of $ONOO^-$ and *S*-nitrosocysteine (Fig. 7).

Subsequently we examined whether inhibition of nitric oxide-induced exocytosis was correlated with reduced NMDA receptor stimulation, and was thus excitotoxicity. Therefore we treated neurons with a combination of botulinum neurotoxin C (which cleaves the plasma membrane-docking protein syntaxin) and tetanus toxin (which cleaves the vesicular docking protein synaptobrevin/VAMP) (Montecucco and Schiavo, 1995). This combination completely prevented $ONOO^-$- and *S*-nitrosocysteine-induced apoptosis (Fig. 8) and significantly reduced the $[Ca^{2+}]_i$ increase triggered by *S*-nitrosocysteine or $ONOO^-$ (Fig. 8). An important role for stimulated synaptic neurotransmitter release in CGC apoptosis was supported by the following observations. (i) Blocking glutamate uptake in otherwise unstimulated cultures with 15 μM *trans*-pyrrolidine-2,4-dicarboxylate (Bridges *et al.*, 1991) resulted in neuron death (84 ± 7% of neurons within 12 h), which was completely blocked by MK-801. In this experimental paradigm of autocrine neuron death due to unstimulated neurotransmitter release, apoptosis of CGC was 5 times slower than that found after the putative triggering of exocytosis by nitric oxide. (ii) Neurons were seeded in small patches (i.e. the total number per culture dish was >100 times lower than normal) to test whether the toxicity of nitric oxide requires a general increase in excitatory neurotransmitter concentrations in the culture medium or whether a local increase, possibly at synaptic clefts, is sufficient. Under these conditions $ONOO^-$ still induced CGC apoptosis, which was prevented by pretreatment with clostridial toxins or MK-801 (89 ± 4 and 98 ± 3% of the neurons survived when patches were pretreated with clostridial toxins and MK-801, respectively).

TABLE 1. Pharmacological modulation of $ONOO^-$/*S*-nitrosocysteine toxicity

Inhibitor	Concentration (μM)	Inhibition of toxicity (% ± SD) by:	
		S-nitrosocysteine (10 μM)	$ONOO^-$ (50 μM)
	1–10	<5	<5
Pyruvate	10 000	<5	<5
Pyruvate + GPT	10 000 plus 25 U/ml	36 ± 4*	42 ± 7*
Cycloheximide	1–50	<5	<5
Actinomycin D	0.1–10	<5	<5
Benzamide	500–2500	10 ± 5 (2.5 mM)	<5
3-Aminobenzamide	500–2500	<5	<5
Nicotinamide	500–2500	<5	n.d.
Superoxide dismutase	1000 U/ml	54 ± 6*	n.d.
N-methyl arginine	30–300	<5	<5

Cerebellar granule cells (9 DIV) were preincubated with the indicated concentrations of inhibitors for 30 min before *S*-nitrosocysteine or $ONOO^-$ was added. Cytotoxicity (determined by MTT reduction after 16 h) was 90% in cells pretreated with solvent controls (CSS or DMSO) only.
n.d., not determined.
*$P \leq 0.05$.

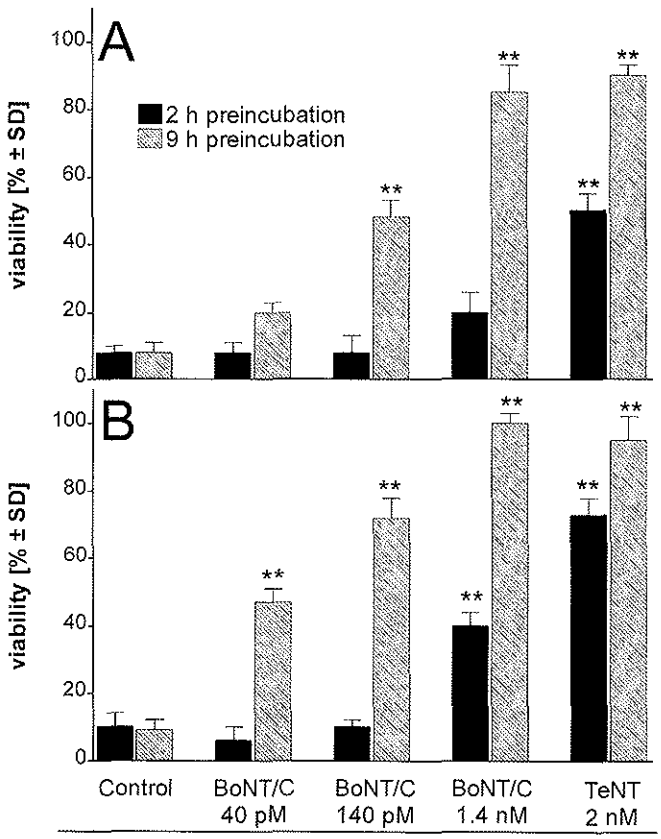
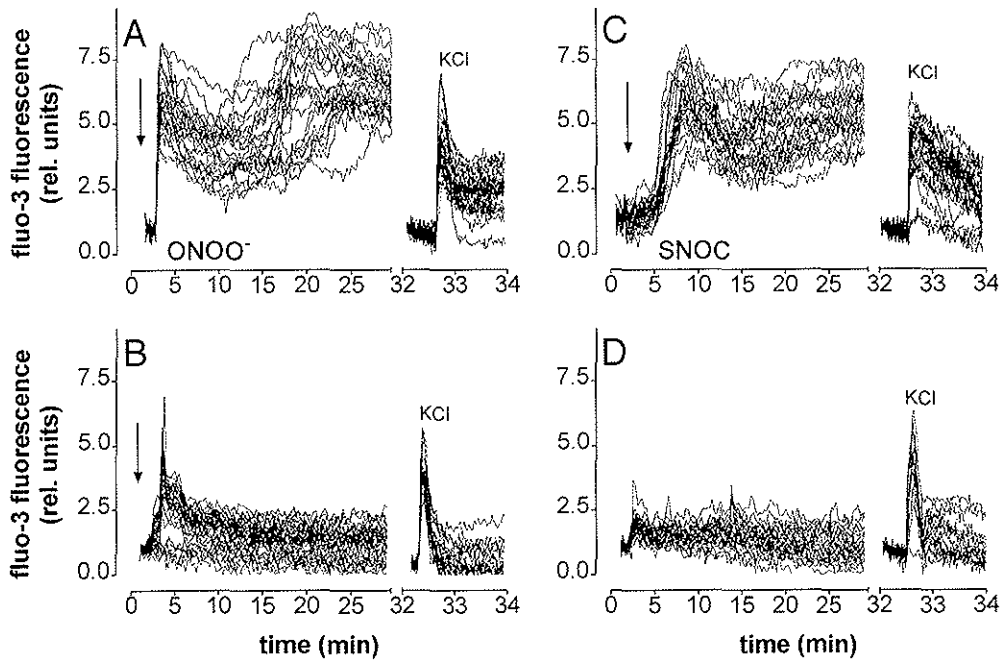
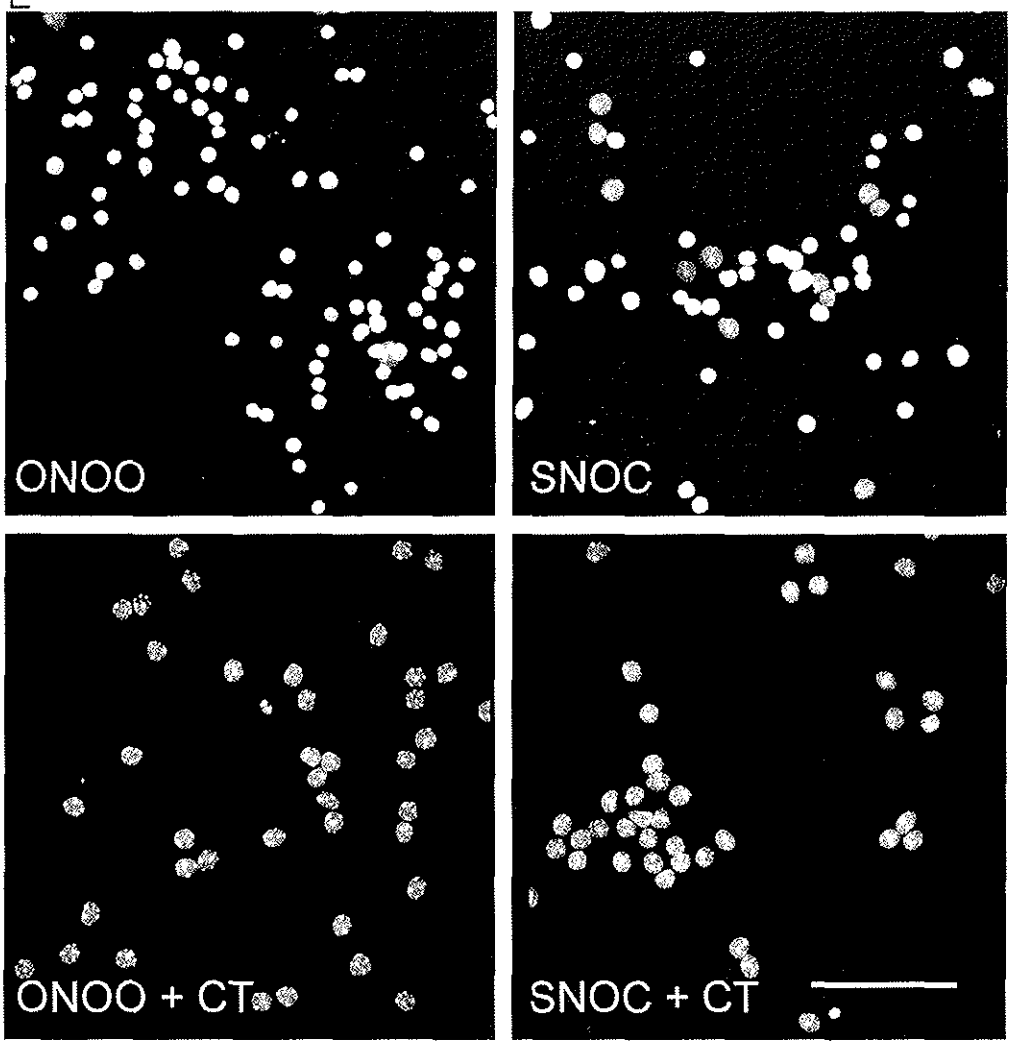


FIG. 7. Protection of CGC from *S*-nitrosocysteine/$ONOO^-$ toxicity by clostridial neurotoxins. CGC were preincubated with clostridial toxins as indicated and then challenged with 10 μM *S*-nitrosocysteine (A) or 80 μM $ONOO^-$ (B). Viability was determined 16 h later by the MTT method. Data are mean ± SD of triplicate determinations. **$P < 0.01$ versus control. BoNT/C, botulinum neurotoxin C.

FIG. 8. Prevention of *S*-nitrosocysteine/ONOO-induced CGC apoptosis and $[Ca^{2+}]_i$ increases by botulinum neurotoxin C plus tetanus toxin. CGC were loaded with fluo-3 for imaging of $[Ca^{2+}]_i$ changes (A–D), or used directly for determination of apoptosis (E). Cultures were challenged directly (A, C, E, top) or pretreated with 500 pM botulinum neurotoxin C plus 5 nM tetanus toxin for 5 h (B, D, E, bottom), and then stimulated with either 50 μM $ONOO^-$ (A, B, E) or with 25 μM *S*-nitrosocysteine (SNOC; C–E). The percentage of apoptosis was determined after staining with H-33342 (E). Clostridial toxins prevented the formation of condensed and hyperfluorescent apoptotic nuclei in neurons challenged with *S*-nitrosocysteine or $ONOO^-$. They also prevented a sustained increase in $[Ca^{2+}]_i$, although occasionally a short, transient rise was observed. After termination of the $[Ca^{2+}]_i$ measurements (A–D) in *S*-nitrosocysteine- or $ONOO^-$ treated cultures, the programme was immediately restarted (initial fluorescence levels set to 1) and changes in $[Ca^{2+}]_i$ due to depolarization with 50 mM K^+ were recorded within the same cultures. Clearly delayed recovery was always observed in *S*-nitrosocysteine-treated neurons (C). Scale bar in E, 50 μm.


A
B
C
D
KCl
ONOO⁻
SNOC
fluo-3 fluorescence (rel. units)
7.5
5.0
2.5
0.0
0 5 10 15 20 25 32 33 34
time (min)

E


ONOO
SNOC
ONOO + CT
SNOC + CT

Discussion

In the present study we showed that exposure to $ONOO^-$ or nitric oxide induced apoptotic cell death in a population of synaptically-linked neurons by stimulating autocrine/paracrine excitotoxicity.

Our interpretation of $ONOO^-$/nitric oxide-induced cell death in CGC cultures as autocrine/paracrine excitotoxicity is based on the following findings. (i) The nitric oxide donors, as well as $ONOO^-$, selectively killed differentiated CGCs, which were also sensitive to NMDA toxicity, but not immature cells, which were insensitive to NMDA toxicity (i.e. during the early days *in vitro*). (ii) Apoptosis was associated with a sustained increase in $[Ca^{2+}]_i$ and was dependent on extracellular Ca^{2+}. The relative increases in $[Ca^{2+}]_i$ were followed in real time in a large number of individual neurons under conditions of different pharmacological pretreatment and with different concentrations of $ONOO^-$ or nitric oxide donors. A sustained $[Ca^{2+}]_i$ increase was observed only under conditions that resulted in neuron death. Similar correlations of cell death with relative changes in $[Ca^{2+}]_i$ have also been observed in other paradigms of excitotoxicity (Choi, 1995). (iii) Apoptosis, as well as the increase in $[Ca^{2+}]_i$, was prevented by blockers of the NMDA receptor. This suggests that Ca^{2+} influx was triggered by stimulation of this receptor. Further, prevention of the $ONOO^-$-induced increase in $[Ca^{2+}]_i$ by the competitive antagonist APV implies that $ONOO^-$ induced the release or facilitated the action of an endogenous receptor agonist. In agreement with electrophysiological data (Fagni *et al.*, 1995), this suggests that the nitric oxide donors did not open NMDA receptor channels directly. Prevention of toxicity by the NMDA receptor antagonists also argues for the causal involvement of NMDA receptor-mediated Ca^{2+} influx in $ONOO^-$/nitric oxide toxicity. (iv) Clostridial toxins that block exocytosis also protected CGC from the increase in $[Ca^{2+}]_i$ and apoptosis elicited by $ONOO^-$/nitric oxide.

The accumulation of a mediator that stimulates the NMDA receptor in this system can be explained either by nitric oxide-stimulated neurotransmitter release (O'Dell *et al.*, 1991; Hirsch *et al.*, 1993; Meffert *et al.*, 1994, 1996; West and Galloway, 1996), e.g. glutamate, or by a possible direct or indirect nitric oxide-induced block of neurotransmitter reuptake (Nicholls and Attwell, 1990). Our experiments with a pharmacological blocker of glutamate reuptake showed that an increase in endogenously released excitatory amino acids is sufficient to induce apoptosis in CGC cultures. However, the more rapid onset of apoptosis and the quick increase in $[Ca^{2+}]_i$ after exposure to $ONOO^-$/nitric oxide support the assumption that stimulated neurotransmitter release rather than just passive accumulation was involved. This conclusion is further supported by the experiments with the clostridial toxins (see below). The experiments in which reduction of the number of neurons did not modify the cytotoxic paradigm observed in normal cultures (i.e. nitric oxide still caused apoptosis which was prevented by NMDA receptor antagonist and clostridial toxins) suggest that cell death here was probably mediated by a specific synaptic mechanism rather than by generalized neurotransmitter release.

Perhaps the best evidence for the involvement of exocytosis in $ONOO^-$/nitric oxide-mediated CGC apoptosis is the effect of the clostridial toxins (Montecucco and Schiavo, 1995). Tetanus toxin and botulinum neurotoxin specifically interfere with the mechanism of vesicle fusion at the presynaptic membrane. In the presence of the clostridial toxins, neurons were consistently and entirely protected from concentrations of $ONOO^-$ or nitric oxide donors that otherwise caused up to 90% apoptosis in CGC cultures.

The chemical nature of the putatively released excitotoxic mediator has not been identified. Partial protection by a glutamate-removing enzyme system suggests the involvement of glutamate itself. As in the excitotoxicity due to exposure of cells to HIV gp120 (Lipton, 1992), hypoxia (Cheng and Mattson, 1992; Monyer *et al.*, 1992a) or increased extracellular Ca^{2+} (Sucher *et al.*, 1991), the contribution of other mediators remains an open question.

In cortical cultures, nitric oxide synthase-containing neurons have been shown to be relatively resistant to the direct toxicity of nitric oxide and its putative metabolite $ONOO^-$ (Koh and Choi, 1988; Dawson *et al.*, 1993). Since CGC express brain nitric oxide synthase, they may also be relatively resistant to toxic mechanisms directly triggered by nitric oxide, such as poly-(ADP-ribose) synthase activation (Zhang *et al.*, 1994). Indeed, we found that *S*-nitrosocysteine concentrations ten times higher than those needed to elicit apoptosis in 95% of all CGC did not affect cultures pretreated with MK-801. Thus, cells which express NMDA receptors should be more sensitive to $ONOO^-$ toxicity than cells in which this receptor subtype is lacking, such as glial and PC-12 cells (Estévez *et al.*, 1995).

In view of these considerations it seems likely that in neurons nitric oxide may exert its toxicity by multiple and eventually synergistic mechanisms. For example, nitric oxide could cause cytotoxicity directly by acting on DNA and proteins in sensitive cells, sensitize certain neuron populations to further excitotoxic challenge, and trigger the release of excitotoxic neurotransmitters. Our observations show that the last mechanism is predominant in CGC cultures. This is in line with findings in different neuronal cells showing that nitric oxide alone, even at concentrations that fully activated guanylate cyclase, was not directly cytotoxic (Lafon-Cazal *et al.*, 1993a; Zinkland *et al.*, 1993; Hewett *et al.*, 1994). Accordingly, endogenous nitric oxide generation from bNOS does not seem to further contribute to the excitotoxic mechanism described here. However, in our conditions the contribution of endogenous nitric oxide generation may be obscured by the presence of saturating concentrations of exogenous nitric oxide/$ONOO^-$. It is conceivable that prolonged activation of the endogenous nitric oxide synthase would otherwise contribute to the demise of CGC (Lafon-Cazal *et al.*, 1993b; Marcaida *et al.*, 1995).

Often, the diverse effects of nitric oxide donors on neuron survival in different culture systems have been explained by the redox state and chemical nature of nitric oxide or its metabolites (Lipton *et al.*, 1993). A special role has been attributed to $ONOO^-$ as a terminal mediator of toxicity, compared with other, less reactive nitric oxide congeners. The present study shows clearly that direct exposure of neurons to $ONOO^-$ does not result in non-specific breakdown of cellular homeostasis leading to immediately lethal events. Rather, $ONOO^-$ seems to act by an indirect but specific mechanism (i.e. by stimulating the exocytosis of excitatory neurotransmitters). The recruitment of a physiological pathway to initiate neuron demise in cells exposed to increasing nitric oxide levels suggests that the threshold between fine-tuning of neuronal responses [i.e. nitric oxide-stimulated neurosecretion (Meffert *et al.*, 1994)] and neuron injury may simply be decided by the nitric oxide/$ONOO^-$ concentration. Accordingly, it has been shown that the mode of cell death also depends on the intensity of the insult (Bonfoco *et al.*, 1995).

In conclusion, pathological situations may be conceived in which imbalances in nitric oxide production and in the release/reuptake of excitatory amino acids would create a vicious circle forming the basis of subsequent neurodegeneration.

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Abbreviations

APV	D-2-amino-5-phosphonovaleric acid
BME	Eagle's Basal Medium
CGC	cerebellar granule cell(s)
CSS	controlled salt solution
DIV	days *in vitro*
EH-1	ethidium homodimer-1
Fluo-3-AM	acetoxymethyl ester of fluo-3
MK-801	(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrasodium bromide
SNAP	*S*-nitroso-*N*-acetyl-penicillamine

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