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Calpain inhibitors prevent nitric oxide-triggered excitotoxic apoptosis

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The pathogenesis of some neurodegenerative disorders has been linked to excitotoxicity, excess generation of nitric oxide (NO) and apoptosis. Here, we used a model of NO-triggered neuronal apoptosis that was strictly dependent on autocrine NMDA receptor (NMDA-R) activation and intracellular Ca^{2+} increase. We investigated the efficiency and potentially beneficial effects of calpain inhibition. Three calpain inhibitors that prevented intracellular fodrin proteolysis also blocked apoptotic features such as decrease in mitochondrial membrane potential, chromatin breakdown, and subsequent death of cerebellar granule neurons exposed to NO donors (S-nitroso-L-glutathione, S-nitroso-N-acetyl-D,L-penicillamine, and diethylamino-diazenolate-2-oxide). Since inhibitors did not interfere with NMDA-R activation, we suggest that block of calpains blunts NO-triggered neuronal apoptosis by stopping the cascade downstream of primary autocrine excitotoxic events. *NeuroReport* 12:3645–3648 © 2001 Lippincott Williams & Wilkins.

Key words: Apoptosis; Calpains; Excitotoxicity; Mitochondria; Nitric oxide

INTRODUCTION

Massive generation of the pleiotropic messenger molecule nitric oxide (NO) has been implicated in many neuropathological conditions including ischemia [1]. NO inhibits the mitochondrial respiratory chain *in vitro* [2], stimulates neurotransmitter release from synaptosomes [3] and can cause autocrine excitotoxicity in neuronal cultures [4,5]. Thus, NO enhances excitotoxic events at different steps.

A cyclic process of self-enhancing loops accounts for NO-mediated neuronal death: impairment of mitochondrial function leads to energy failure [2,6]. This impairs ion homeostasis, which leads to plasma membrane hypopolarization. In turn, hypopolarization removes the Mg^{2+}-dependent block of the NMDA receptor (NMDA-R) [7] that becomes hypersensitive towards stimulation. NMDA-R-mediated Ca^{2+} increase then can elevate energy demand, enhance depolarization, trigger further Ca^{2+} increase and favor the release of endogenous glutamate. This putatively self-propagating process finally results in the disruption of intracellular Ca^{2+} homeostasis and excitotoxicity [7].

We have previously established that NO and other mitochondrial inhibitors trigger a NMDA-R-dependent apoptosis mediated by caspases in cerebellar granule cells (CGC) [5,8,9]. Besides caspases, Ca^{2+}-dependent cysteine proteases, calpains, have also been implicated in neuronal apoptosis [10]. Thus, the question that is addressed here is whether activation of this class of proteases can link NO-triggered excitotoxic events and apoptosis execution.

MATERIALS AND METHODS

Cell culture: Murine CGC were isolated from 8-day-old specific pathogen free BALB/e mice obtained from the Animal Unit of the University of Konstanz. They were cultured as described [8]. Briefly, dissociated neurons were plated on poly-L-lysine coated dishes at a density of about 0.25×10^6 cells/cm^2 and cultured in Eagle's basal medium (BME; Gibco, Life Technologies, Germany) supplemented with 10% heat-inactivated fetal calf serum, 20 mM KCl, 2 mM L-glutamine and 1% penicillin-streptomycin, and cytosine arabinoside (10 μM, added 48 h after plating). Neurons were used without further medium changes at 8–10 days *in vitro.* Cultures were exposed to the NO donors S-nitroso-L-glutathione (GSNO), S-nitroso-N-acetyl-D,L-penicillamine (SNAP), and diethylamino-diazenolate-2-oxide (DEA-NO) in their own medium. The culture medium was exchanged for a controlled salt solution (CSS; 120 mM NaCl, 25 mM HEPES, 25 mM KCl, 1.8 mM $CaCl_2$, 4 mM $MgCl_2$, 15 mM glucose) 4 h after the start of the incubation, and the cells were left in this medium (usually for 20 h) without re-addition of any inhibitors. All inhibitors including the NMDA-R antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine (MK801) from Biotrend (Köln, Germany) were added 30 min before exposure to NO donors.

Cytotoxicity assays: To assess plasma membrane integrity and nuclear morphology, CGC were double stained with the chromatin dyes SYTOX (0.5 μM, non-cell per-

meable, green-fluorescent) and H-33342 (1 μg/ml, cell permeable, blue-fluorescent). All dyes were obtained from Molecular Probes (Eugene, OR, USA). Apoptosis was characterized by scoring condensed and highly fluorescent nuclei. About 500 cells were counted in three different fields in three different culture wells, and experiments were repeated in at least three different preparations. In addition, the percentage of viable cells was quantified 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.

Mitochondrial function: The mitochondrial membrane potential ($\Delta\Psi$) was monitored in cells loaded with 5 nM tetramethylrhodamine ethyl ester (TMRE, $\lambda_{ex} = 568$ nm, $\lambda_{em} = 590$ nm) from Molecular Probes for 10 min. The fluorescence of neurons treated with the mitochondrial poison carbonyl cyanide chlorophenylhydrazone (50 μM) was used as reference for depolarized mitochondria [9]. Measurement of ATP was performed luminometrically after lysing cells in ATP-releasing agent (Sigma, Deisenhofen, Germany) with a commercial kit (Boehringer-Mannheim, Mannheim, Germany) according to the supplier's instructions.

Ca^{2+} measurements: Changes of the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by imaging individual neurons loaded in their original medium with 1 μM fluo-3 acetoxymethyl ester (fluo-3-AM, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm) from Molecular Probes for 10 min as described previously [8]. After loading, the medium was exchanged for CSS for 5 min to allow complete de-esterification of fluo-3. The buffer was exchanged again for the original neuron-conditioned, complete BME medium supplemented with 20 mM HEPES. CGC were allowed to equilibrate at room temperature for 10 min before exposure to GSNO. Relative mean fluorescence levels from 10–20 neurons were recorded by video microscopy at 20 s intervals over 20 min. The mean fluorescence level of each marked cell was arbitrarily set to 1 at the beginning of each experiment.

Enzymatic assays: Enzymatic activities of purified rabbit muscle calpain (0.75 U/ml), recombinant caspase-2 (3 ng/ml) or caspase-3 (3 ng/ml) were measured by a kinetic assay based on the cleavage of LLVY-aminomethylcoumarine (LLVY-amc, Bachem, Heidelberg, Germany) by calpain, of VDVAD-aminotrifluoromethylcoumarine (VDVAD-afc, California Peptide Research, Napa, CA, USA) by caspase-2 or DEVD-afc (Biomol Hamburg, Germany) by caspase-3 described previously [8]. Briefly, enzymes were added to serial dilutions of the inhibitor Ac-Leu-Leu-L-norleucinal (calp I), Ac-Leu-Leu-L-methional (calp II), z-Val-L-phenylalaninal (calp III) or z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) (all from Bachem) and incubated for 10 min at room temperature. Release of afc ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 508$ nm) or amc ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 442$ nm) was monitored and activity was calibrated with afc or amc standard solutions.

Electrophoretic assays: Fodrin proteolysis was analyzed by immunoblot with anti-fodrin monoclonal antibody (clone 1622, 1:1000) from Chemicon (Temecula, CA, USA) as described previously [8]. Field inversion gel electrophoresis was performed as described previously [11]. About 5×10^6 cells were embedded into 40 μl agarose blocks. As molecular weight markers, λ DNA concatemers of $n \times 50$ kilobase pairs (kbp) were used.

RESULTS

NO-induced apoptosis and mitochondrial failure require NMDA-R activation: Exposure of CGC to different NO donors (DEA-NO, GSNO, SNAP) induced apoptotic changes including nuclear chromatin condensation (Fig. 1a, inlay), DNA fragmentation and phosphatidylserine exposure [8]. NMDA-R antagonists such as MK801 prevented NO-triggered Ca^{2+} increase (Fig. 1b) and apoptosis (Fig. 1a). To examine long-term survival, the cell culture medium was replaced 4 h after NO challenge by normal conditioned medium. CGC pretreated with MK801 survived for >48 h without any morphological signs of toxicity or lost capacity to reduce MTT (data not shown). Neurons exposed to NO alone for 4 h showed condensed

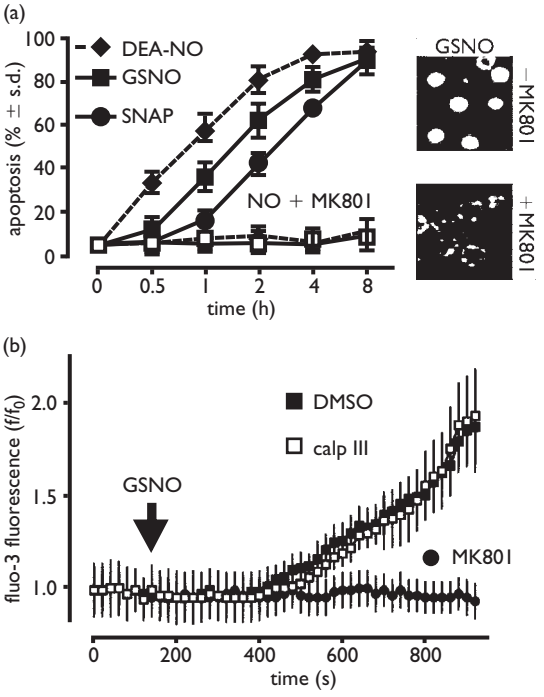

Fig. 1. NO mediates apoptosis in neurons via excitotoxic mechanisms. (**a**) CGC were incubated with 50 μM DEA-NO, 200 μM GSNO or 500 μM SNAP in the absence or presence of 2 μM MK801. At the times indicated (logarithmic scale), neurons were stained with H-33342, and apoptosis is expressed as the percentage of nuclei with condensed chromatin. Data are means ± s.d. of triplicate determinations. Inlays show individual neuronal nuclei treated with 200 μM GSNO in the absence or presence of MK801 stained with H-33342 after 4 h (image width corresponds to 20 μm). (**b**) CGC were loaded with fluo-3-AM and pretreated with solvent control (DMSO, 0.4% dimethylsulfoxide), 2 μM MK801 or 100 μM calp III before they were challenged with 500 μM GSNO. The increase of $[Ca^{2+}]_i$ over time (fluo-3 fluorescence $[f/f_0]$) was followed by digital video imaging. Data are means ± s.e.m. from 10–20 individually recorded neurons.

chromatin and lysed within the following 4–8 h regardless of medium changes.

Since mitochondrial failure is one of the early features of excitotoxicity [12,13] we examined the effect of NMDA-R inhibition on NO-dependent alterations of mitochondrial function. GSNO treatment caused an early and complete loss of $\Delta\Psi$ (Fig. 2a, middle panel) which preceded nuclear condensation (not shown). Pretreatment with MK801 prevented the NO-induced decrease of $\Delta\Psi$ (Fig. 2a, right panel), suggesting that the $\Delta\Psi$ loss was due to excitotoxic processes downstream to the NMDA-R-induced Ca^{2+} increase.

In analogy with other models of excitotoxicity [11], the loss of $\Delta\Psi$ was linked to intracellular ATP depletion. GSNO (200 μM) triggered a substantial ATP decline starting at 60 min. Within 2 h 80 ± 5% ATP was lost and recovery was not observed. Notably, energizing mitochondria by the addition of methylsuccinate (5 mM) as complex II substrate prevented GSNO-induced loss of $\Delta\Psi$ and delayed apoptosis and ATP depletion (data not shown).

Calpain inhibitors prevent NO-induced excitotoxicity: To determine intracellular calpain activity in neurons challenged with NO, we studied cleavage of the calpain substrate fodrin. We observed the typical calpain-mediated cleavage pattern of fodrin (non-erythroid α-spectrin) into fragments of about 150 kDa [10] in NO-treated neurons (Fig. 2b). Fodrin degradation did not occur when neurons were pretreated with calpain inhibitors (calp I, calp II, calp III; Fig. 2b) and neurons were protected from GSNO-induced apoptosis (Fig. 3a). The effect was concentration dependent (Fig. 3a) and correlated with the IC_{50} for inhibition of fodrin proteolysis [9]. Complete protection, i.e. 48 h survival, was still observed when inhibitors were added up to 15 min after NO (data not shown).

It was shown previously that caspases are involved in NO-induced neuronal apoptosis [8]. Therefore, we tested the three different calpain inhibitors for their ability to inhibit the execution caspase-2 and -3 in comparison with the pan-caspase inhibitor zVAD-fmk. Two of these calpain inhibitors (calp I and II), which protected CGC from NO-induced apoptosis, did not inhibit caspase activity (Table 1).

Next, we investigated whether calpain inhibitors could influence the NMDA-R-mediated Ca^{2+} increase. Neither the Ca^{2+} influx through the NMDA-R [9] nor the initial $[Ca^{2+}]_i$ increase triggered by NO donors was affected by

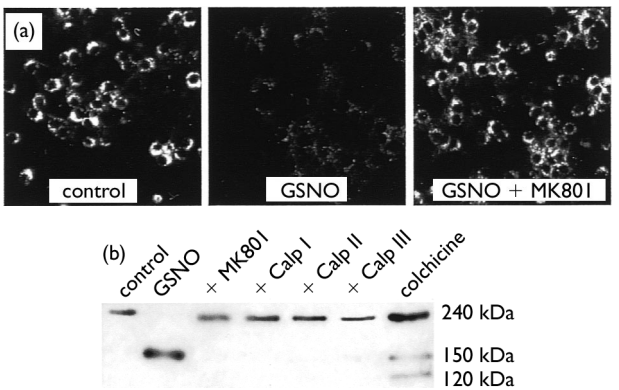

Fig. 2. GSNO-mediated loss of mitochondrial $\Delta\Psi$ and fodrin proteolysis. (**a**) CGC were challenged with 200 μM GSNO in the absence or presence of 2 μM MK801. After 2 h neurons were stained with 5 nM TMRE (mitochondrial $\Delta\Psi$). Samples were imaged by confocal microscopy. Image width = 90 μm. (**b**) CGC were incubated with 2 μM MK801, calpain inhibitors (100 μM calp I, 200 μM calp II, 100 μM calp III) or solvent control (DMSO) only and challenged with 200 μM GSNO. After 4 h incubation (< 5% of CGC had lost membrane integrity), cells were lysed and fodrin proteolysis was analysed by immunoblot. As positive control for fodrin proteolysis, the proapoptotic toxin colchicine [22] was used. Colchicine treatment (1 μM, 12 h) produced proteolytic fragments of 150 and 120 kDa, whereas NO produced 145/150 kDa fragments.

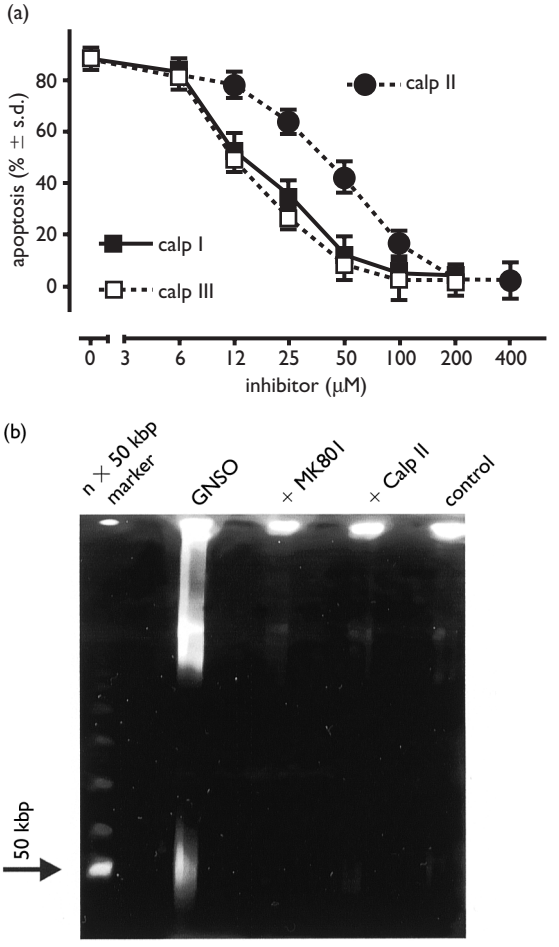

Fig. 3. Calpain inhibitors prevent GSNO-triggered apoptosis. (**a**) CGC were incubated with various calpain inhibitors at concentrations indicated (logarithmic scale) and challenged with 200 μM GSNO. After 20 h neurons were stained with H-33342, and apoptosis is expressed as percentage of nuclei with condensed chromatin. Non-condensed dead cells (SYTOX positive) were not observed in this model. Data are means ± s.d. of triplicate determinations. (**b**) CGC were incubated with 2 μM MK801, 200 μM calp II or solvent control (DMSO) only and challenged with 200 μM GSNO. After 4 h incubation (< 5% of CGC had lost membrane integrity), DNA fragmentation was analyzed by field inversion gel electrophoresis. The arrow indicates high mol. wt DNA fragments of 50 kbp.

Table 1. Specificity profile of calpain inhibitors used to prevent NO-triggered apoptosis.

IC_{50} (μM)	calp I	calp II	calp III	zVAD-fmk
calpain	0.2	0.2	0.1	>100
caspase-2	>100	>100	>100	0.4
caspase-3	>50	>100	18	0.2

Serial dilutions of calpain inhibitors (calp I, calp II and calp III) or the caspase inhibitor zVAD-fmk were preincubated for 10 min with purified muscle calpain, recombinant caspase-2 or caspase-3. Enzyme activity was measured by a kinetic assay based on the cleavage of LLVY-amc for calpain, VDVAD-afc for caspase-2 or DEVD-afc for caspase-3. IC_{50} values were obtained by 4-parameter fit of the inhibition curves.

calpain inhibitors (Fig. 1b), indicating that the inhibitors act downstream of the NMDA-R. In accordance with this, NO-induced fodrin cleavage was prevented by NMDA-R block (Fig. 2b), showing that the intracellular proteolysis was a downstream effect of NMDA-R-mediated Ca^{2+} overload. Calpain inhibitors also prevented GSNO-mediated DNA fragmentation to a similar extent as the NMDA-R antagonist MK801 (Fig. 3b). These findings suggest that calpain activation downstream to the Ca^{2+} overload results in apoptotic chromatin breakdown. Since the loss of $\Delta\Psi$ triggered by NO was completely, and persistently prevented by calpain inhibitors (data not shown), these agents interfere with execution of cell death upstream to mitochondrial failure.

DISCUSSION

Neurons exposed to NO undergo apoptosis mediated by mitochondrial failure, Ca^{2+} increase, and calpain activation. Although all these events are strictly dependent on NMDA-R activation, the primary biochemical effect of NO may be inhibition of mitochondrial complex I [2,6]. The resulting functional impairment may facilitate excitotoxicity [1,14,15], by sensitizing neurons towards glutamate [7] and priming release of excitotoxic mediators [16].

The downstream mechanisms in NO-mediated excitotoxic apoptosis may involve both calpains and caspases. In a complex interrelationship calpains may activate caspases [17], which may then cleave the endogenous calpain inhibitor calpastatin [18]. Eventually, strong activation of calpains can destroy caspases [19], e.g. in excitotoxic death [20]. Fodrin is cleaved by both caspases and calpains. The typical fodrin cleavage pattern by calpain leads to the formation of ~150 kDa fragments [10], as observed here in NO-triggered CGC apoptosis. Caspase-3, the executioner protease considered essential for caspase-dependent neuronal apoptosis [21], generates a 120 kDa proteolytic fragment [10]. Such a signature cleavage was observed in CGC undergoing apoptosis induced by colchicine (Fig. 2b), a caspase-3-dependent death model [22], but not after NO treatment. Since caspase inhibitors devoid of calpain inhibitory activity also block formation of the 150 kDa proteolytic fragments [8] it is conceivably that caspases other than caspase-3 might function in concert with calpains in this model. Because calpain inhibitors blocked all examined downstream death events without affecting NO-dependent initial NMDA-R activation, calpain-mediated proteolytic activity links NO-induced Ca^{2+} influx with execution of cell death.

CONCLUSION

Our study establishes that inhibition of calcium-activated proteases (calpains) prevents excitotoxic events downstream of the increase in cytosolic calcium due to NMDA-R overstimulation. This suggests that calpains act upstream of the mitochondrial disruption caused by calcium overload. Since caspase inhibitors have previously been shown to prevent apoptosis elicited by indirect excitotoxicity, inhibition of the two classes of proteases may be an effective strategy to reduce cell death in cerebral ischemia and other pathological conditions linked to NMDA-R activation.

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