Nitric oxide damages neuronal mitochondria and induces apoptosis in neurons

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Abstract The cytotoxic effect of nitric oxide on primarily cultured rat cerebellar granule cells was studied, and the mechanisms were discussed. The results showed that nitric oxide donor S-nitroso-N-acetyl-penicillamine (SNAP; 500 μmol/L) could induce apoptosis in immature cultures of cerebellar granule cells. Flow cytometry and HPLC analyses revealed that after treatment with SNAP, the mitochondrial transmembrane potential and the cellular ATP content decreased significantly. Nitric oxide scavenger hemoglobin could effectively prevent the neuronal mitochondria from dysfunction and attenuate apoptosis. The results suggested that nitric oxide activated the apoptotic program by inhibiting the activity of mitochondrial respiratory chain and thus decreasing the cellular ATP content.

Keywords: cerebellar granule cells, apoptosis, nitric oxide, mitochondria.

Recent studies have revealed that nitric oxide (NO) is an important biological messenger molecule, which exerts diverse physiological and pathological functions such as blood flow regulation, signal transduction and immune defense[1]. In the mammalian central nervous system, NO plays important roles in neurotransmission and memory, while excessive NO shows neurotoxicity and induces apoptosis in several types of neurons. Under some pathological conditions such as ischemic brain injury, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD) and various forms of cerebellar degeneration, certain types of neurons undergo excessive apoptosis[2,3], and NO-induced neuronal injury might be one of the pathogens. In this regard, studying the mechanism of NO-induced apoptosis may be helpful in understanding apoptosis and neurodegenerative processes more completely, which has both theoretical and practical meanings. Among the many pathways involved in NO-induced neuronal injury, the relationships and interactions between NO and excitatory amino acid receptors (e.g. N-methyl-D-aspartate receptor, NMDA-R) aroused much attention[4], while NO-induced apoptosis through inactivating the mitochondria has not been reported yet. In this study, immature primary cultures of rat cerebellar granule cells, a relatively homogenous culture system of neurons, were employed to investigate the neurotoxicity of NO. The alteration of mitochondrial function during the apoptotic process was studied by flow cytometry and HPLC. The possible pathways involved in NO-induced neuronal damage were discussed.

1 Materials and methods

(ⅰ) Materials. Seven-day-old Wistar rats were purchased from Beijing Medical University. Cell culture plastic ware was purchased from Corning Costar. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, trypsin (1: 250), proteinase K and RNase A were products of Gibco BRL. Agarose, S-nitroso-N-acetyl-penicillamine (SNAP), Thiazolyl blue (MTT), poly-L-lysine and hemoglobin (Hb) were purchased from Sigma. Bovine serum albumin (BSA), ATP and DNase I were purchased from Boehringer Mannheim. Rhodamine 123 (Rh123) was purchased from Molecular Probes. Other reagents made in China were of analytical grade.

(ⅱ) Cell culture. Primary cultures of rat cerebellar granule cells were prepared following procedures described previously[5]. Cells were plated on 6-well multidishes (2×10^6 cells/mL, 2 mL/well) or 24-well multidishes (2.5×10^6 cells/mL, 0.4 mL/well) previously coated with poly-L-lysine. Culture medium consisted of DMEM supplemented with KCl (19.6 mmol/L), glutamine (2 mmol/L), HEPES (10 mmol/L) and heat-inactivated fetal bovine serum (10%, *v/v*). Cells were maintained at 37℃ in a humidified 5% CO_2-95% air atmosphere. Experiments were carried out 48 h after plating. NO donor SNAP (500 μmol/L, final concentration) was added to cerebellar granule cells and the cells were

cultured for indicated time. In some experiments, Hb (5 μmol/L, final concentration) was added to the cells 15 min before the treatment with SNAP.

(iii) Cell viability. Cell viability was assessed by MTT assay[5].

(iv) Morphological studies. Ultrastructure of cells was observed by transmission electron microscope (TEM)[5].

(v) Analysis of DNA fragmentation. The laddering pattern of DNA fragmentation, a well-known characteristic of apoptosis, was detected by agarose gel electrophoresis[5].

(vi) Determination of mitochondrial transmembrane potential. Rh123 is a mitochondrial energization-sensitive fluorescence probe; its fluorescence intensity is directly related to the mitochondrial transmembrane potential[6]. Cells were treated with 0.02% trypsin-20 μg/mL DNase I, washed twice with phenol red-free DMEM containing 0.2% BSA, stained with 1 μmmol/L Rh123 at 37℃ for 25 min, and analyzed with a flow cytometer with 488 nm excitation and 520 nm emission.

(vii) Determination of cellular ATP content. Cellular ATP content was quantified by reverse-phase HPLC[7]. Column: Zorbax Rx-C18 column, 5 μm, 250×4.6 mm i.d.; mobile phase: 0.05 mol/L phosphate buffer (pH 6.0) containing 3.75% methanol, 0.8 mL/min; detection wavelenth: 254 nm.

2 Results

(i) Cell viability. Results of MTT assay indicated that 500 μmol/L SNAP induced time-dependent cell death in cerebellar granule cells (fig. 1), and nitric oxide scavenger hemoglobin effectively protected cells from death.

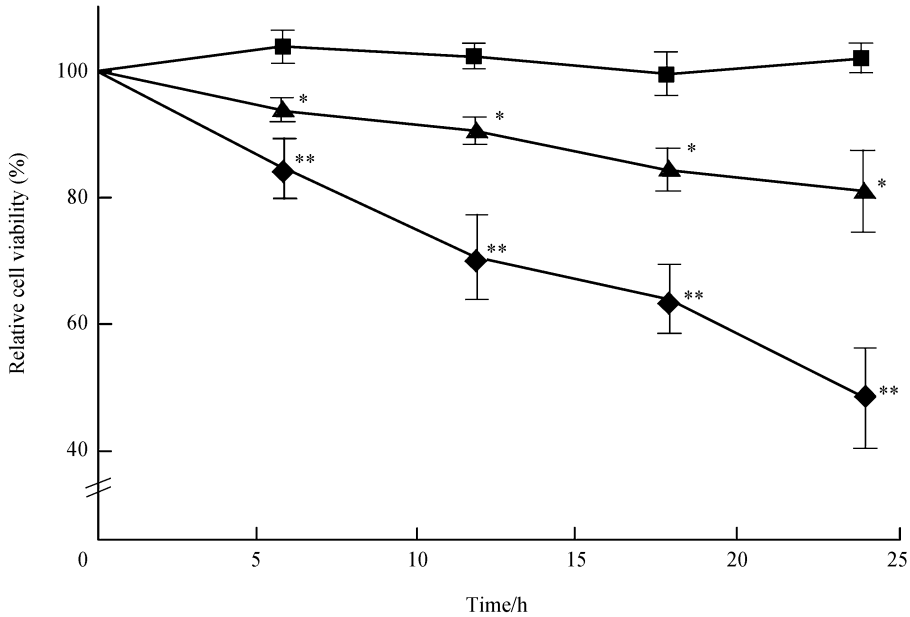

Fig. 1. Cell death in cerebellar granule cells induced by nitric oxide. Cells were incubated with 500 μmol/L SNAP for indicated time and the viability was assessed by MTT assay. ■, normal cells; ◆, cells treated with 500 μmol/L SNAP; ▲, cells treated with 5 μmol/L Hb + 500 μmol/L SNAP. * P< 0.01 in comparison with cells treated with 500 μmol/L SNAP; ** P < 0.01 in comparison with normal cells. Data are mean ±SD, n = 6.

(ii) Characteristics of apoptosis. After the addition of 500 μmol/L SNAP, cerebellar granule cells died gradually, which was characterized morphologically by neurite breakdown, cell shrinkage, and the increase of detached cells. TEM observation revealed that 24 h after the addition of 500 μmol/L SNAP, most of cerebellar granule cells underwent morphological alteration such as chromatin condensation, and apoptotic bodies were formed by the fragmentation of nuclei (fig. 2). Agarose gel electrophoresis of DNA extracted from cells treated with SNAP for 24 h showed a 180 bp ladder

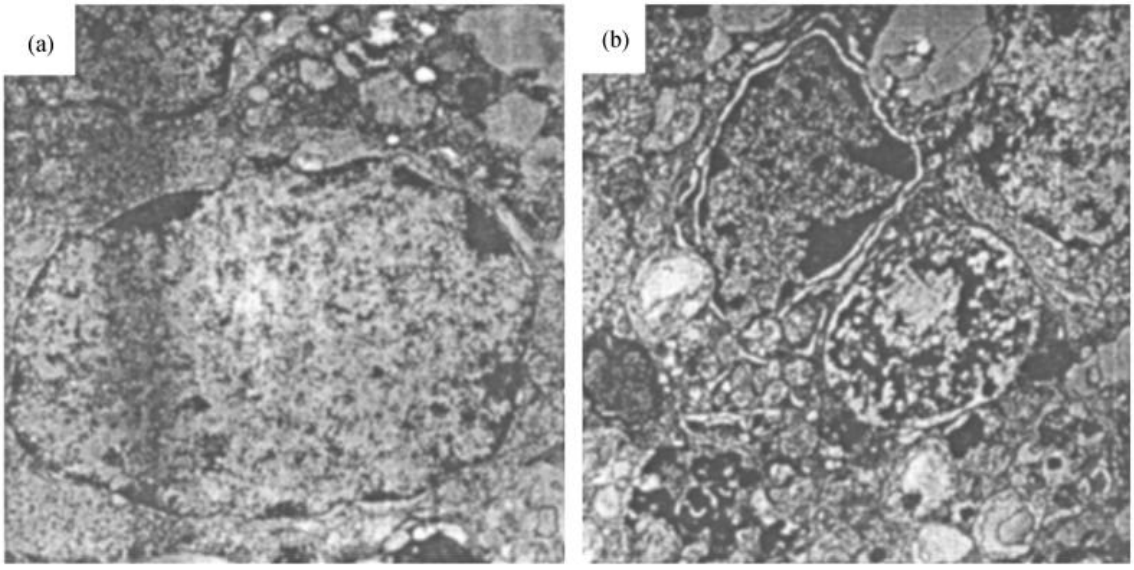

Fig. 2. Nuclear ultrastructure of normal cerebellar granule cells ((a)×10 000) and cerebellar granule cells exposed to 500 μmol/L SNAP for 24 h ((b)×3 000).

pattern (fig.3), indicating the nucleosomal fragmentation of DNA and the formation of oligonucleosomes. The evidence described above indicates that under these experimental conditions, nitric oxide-induced cell death was apoptosis. Pretreating cells with Hb prevented cell morphology from alteration and protected DNA from fragmentation effectively.

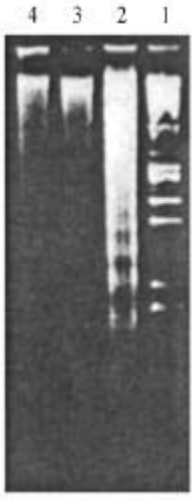

Fig. 3. Nitric oxide-induced DNA nucleosomal fragmentation in cerebellar granule cells. Lane 1, DNA marker (λDNA/*Eco*RI + *Hin*d Ⅲ); lane 2, cells exposure to 500 μmol/L SNAP for 24 h; lane 3, normal cells; lane 4, cells exposed to 5 μmol/L Hb + 500 μmol/L SNAP for 24 h.

(ⅲ) Damage of mitochondria. Results of flow cytometry indicated that exposure to 500 μmol/L SNAP for 3 h caused significant decrease in Rh123 fluorescence intensity, suggesting the decrease of mitochondrial transmembrane potential. HPLC analysis indicated that exposure to 500 μmol/L SNAP for 3 h caused marked decrease in cellular ATP content. These suggested that NO donor SNAP damaged the neuronal mitochondria. Hb, the specific scavenger of NO, effectively prevented the mitochondria from damage (fig. 4).

3 Discussion

Because of its active biological character, nitric oxide, a "double-edge sword", acts as both physiological messenger and cytotoxic agent in the central nervous system. Recent studies showed that NO induced apoptosis in several types of neurons, while the exact mechanisms are still at issue. In order to exclude the involvement of excitatory amino acid receptors, immature cultures of rat cerebellar granule cells (2 days *in vitro*) were employed in the present experiments. This kind of culture consists of relatively homogenous neurons (＞90 %) and the NMDA-R on the cell membrane is immature, and excitatory amino acids (such as NMDA and glutamate) only trigger slight neurotoxicity.

The results of MTT assay indicated that addition of NO donor SNAP triggered cell death in cerebellar granule cells. After exposure of cells to SNAP for 24 h, the neurons showed chromatin condensation and nuclei fragmentation, and apoptotic bodies and the nucleosomal fragments of DNA were formed, which were typical characteristics of apoptosis. These pieces of evidence indicated that

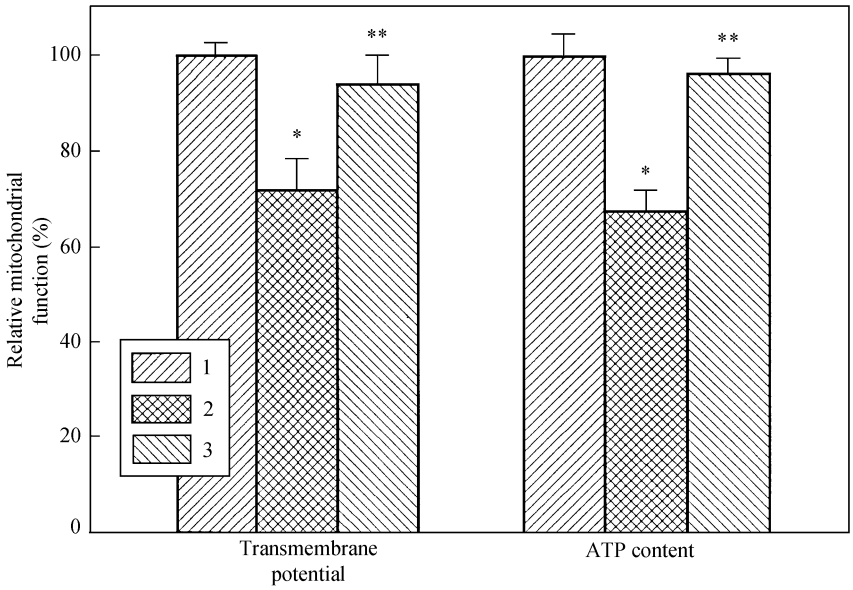

Fig. 4. Nitric oxide-induced mitochondria dysfunction in cerebellar granule cells. After incubation with 500 μmol/L SNAP for 3 h, the mitochondrial transmembrane potential and the cellular ATP content were determined. 1, Normal cells; 2, cells exposed to 500 μmol/L SNAP for 3 h; 3, cells exposed to 5 μmol/L Hb + 500 μmol/L SNAP for 3 h. * $P < 0.01$ in comparison with normal cells; ** $P < 0.01$ in comparison with cells exposed to 500 μmol/L SNAP for 3 h. Data are mean ± SD, $n = 3$.

under these experimental conditions, neuronal cell death induced by SNAP was apoptosis. NO scavenger Hb protected cells from death, suggesting that it was NO that caused the apoptosis of neurons.

Further study revealed that after exposure to SNAP for 3 h, the mitochondrial transmembrane potential and the intracellular ATP content of cerebellar granule cells decreased significantly, and Hb showed protective effect, suggesting that it was NO that caused the dysfunction of mitochondria. Actually, NO can diffuse across the cell membrane and the mitochondrial membrane, react with thiol groups, iron-sulfur clusters and heme proteins and thus inactivate enzymes including complex I, complex II-III, and complex IV of the mitochondrial electron transport chain[8,9]. The inactivation of mitochondrial enzymes leads to the decrease of the mitochondrial energy status, which was proved by the decrease of mitochondrial transmembrane potential. The inactivation of mitochondrial enzymes also caused the decrease in cellular ATP content, which triggered apoptotic program[10]. Blockage of mitochondrial respiration may cause the formation of endogenous superoxide anion[11], which reacts with NO at very high rate ($k \geqslant 6.7\times10^9$ L • mol^{-1} • s^{-1}) to form peroxynitrite, a potent oxidant. Peroxynitrite might cause irreversibly oxidative damage in neuronal cells[5]. It must be emphasized that the cell model used in the present study was immature cultures of rat cerebellar granule cells. This model could effectively exclude the involvement of excitatory amino acid receptors, but the excitation characteristics, the ion channels, and the membrane receptors of these cells are different from mature neurons. So the results and the conclusion of present study could not be applied in mature neuronal culture models and *in vivo* models without further examination. However, in mature neuronal culture models and *in vivo* models, nitric oxide might induce neuronal injury via several pathways such as inactivation of mitochondria, stimulating the release of neurotransmitters, and changing the permiability of ions.

In neuronal cells, maintaining the normal function of mitochondria is very important. When the mitochondria were damaged, the cellular energy status would decrease. The decrease in cellular energy status might cause the depolarization of neuronal membrane and thus trigger a series of signals including Ca^{2+} influx, and lead to neuronal cell death finally. The neuronal cell death caused by mitochondrial dysfunction might be the common pathogen of many neuronal diseases[12]. The present

study indicated that in immature cultures of cerebellar granule cells, nitric oxide damaged the mitochondria, and caused apoptosis finally. This result might have further implication in a more complete understanding of apoptosis and neurodegenerative processes, which will lead to selective methods to control them.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 39800035).

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(Received February 11, 1999)