Nitric Oxide Donor Sodium Nitroprusside Induces Neurotoxicity in Cerebellar Granular Cell Culture in Rats by an Independent Mechanism from L-Type or Dantrolene-Sensitive Calcium Channels

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The effects of sodium nitroprusside (SNP) in rat cerebellar granular cell culture were investigated in the present study. All doses of the SNP (10, 25, 50, 100, 250, 500 μM) were able to induce cell death compared with control values ($p<0.001$ for all groups tested). Interestingly enough, a nonlinear dose–response curve was obtained for SNP-induced neurotoxicity. We also investigated the possible neuroprotective effects of nimodipine and dantrolene, alone or in combination. Both drugs failed to prevent neuronal cell death at the doses tested, either alone or in combination. Despite the fact that the most effective dose was a dantrolene concentration of 10 μM with SNP 500 μM and a concentration of 1 μM with SNP 50 μM, the differences were insignificant statistically. According to our results, SNP-induced cerebellar toxicity appears to be an independent reaction from L-type or endoplasmic reticulum calcium currents.

Key words sodium nitroprusside; dantrolene; nimodipine; neurotoxicity; cerebellum; cell culture

Nitric oxide (NO) plays a dual role (neuroprotection and neurotoxicity) in cerebral ischemia. As a neurotoxin, NO may mediate the ischemic excitotoxic brain injury induced by glutamate release and NMDA receptor overactivation. On the other hand, as a signalling molecule, NO may induce an increase in blood perfusion of ischemic penumbra in the early stages of cerebral ischemia.[1)] NO is also a signalling molecule involved in events crucial to neuronal cell function such as neurotransmitter release, gene transcription and neurotoxicity.[2)] The mechanisms proposed for NO-mediated neurotoxicity include inactivation of the mitochondrial respiratory chain,[3)] *S*-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase,[4)] inhibition of *cis*-aconitase,[5)] activation of poly (ADP-ribose) synthase, and DNA damage,[6)] most of which can be mediated by the formation of nitroso compounds by cellular components. An important role for intracellular calcium in oxidant-induced cell death has been suggested by some,[7)] yet this proposal remains controversial. On the one hand, it has been suggested that an increase in free Ca^{2+} is nothing more than a late event associated with loss of membrane integrity and does not contribute appreciably to oxidant-induced cell death.[8)] On the other hand, it has been proposed that an early increase in intracellular free Ca^{2+} exacerbates oxidative stress, damages mitochondria, activates Ca^{2+}-dependent degradative enzymes, and disrupts the cytoskeleton, all of which play a central role in oxidant-induced cell death.[9)] The endoplasmic reticulum is the major intracellular Ca^{2+} storage site.[8)] Oxidants, sulfhydryl active agents, and free radicals also inhibit Ca^{2+}-ATPases and release Ca^{2+} from the endoplasmic reticulum, suggesting that endoplasmic reticulum Ca^{2+} could play a role in oxidant toxicity.[10)]

The present study investigates the possible role of intracellular and extracellular Ca^{2+} currents in sodium nitroprusside (SNP)-induced neurotoxicity in rat cerebellar granular cell cultures.

MATERIALS AND METHODS

Primary cultures of cerebellar granule cells were prepared from 1-d-old newborn Sprague–Dawley rats as previously described by Xu and Wojcik.[11)] Briefly, newborn rats were decapitated and the cerebellum was removed. It was suspended in 5 ml of calcium-free Hank's balanced salt solution (HBSS, Sigma Chemical, St. Louis, MO, U.S.A.) containing 2 ml of trypsin–EDTA (0.25% trypsin–0.02% EDTA, Biol Ind, Haemek, Israel) at 37 °C for 40 min. Trypsin digestion was ended adding by 10 ml of HBSS containing deoxyribonuclease type 1 (120 unit/ml, Sigma Chemical). After 3 min of centrifugation at 800 rpm, the resulting pellets were suspended. The bases of 25 cm^3 polypropylene tissue culture flasks were covered with poly-D-lysine. Twenty-four hours before the experiment, poly-D-lysine 0.1 mg/ml, 30000—70000 MW, was dissolved in phosphate-buffered saline (PBS) and the flask bases were filled with the same solution. After standing 5 min at room temperature, the solution was vacuumed and left to dry in a laminar flow bench overnight. Cell suspensions 0.2 ml were plated in 2.5 ml of medium that contained 10% fetal calf serum (FCS, Biol Ind) and basal Eagle's Medium without antibiotics. After 30 min, the media were changed to eliminate nonadhering cells and fresh HBSS was added to the flasks. The culture dishes were kept at 37 °C in humidified 95% air and 5% CO_2. After 24—48 h, cytosine arabinoside (cytosine 1-β-D-arabinofuranoside) 10 μM was added to the culture medium to prevent the proliferation of nonneuronal cells. Culture media were changed twice weekly, and neurons were used for neurotoxicity experiments following 8—10-d *in vitro* incubation. Each experimental group was tested in five culture media ($n=5$) and at least 15 microscopic areas were counted for each medium tested.

SNP (Schwartz Pharma, Mannheim, Germany) dissolved in sodium citrate was added to culture flasks at concentration of 10, 25, 50, 100, 250 and 500 μM. Two of the most toxic doses of SNP were also tested with nimodipine (Bayer, Turkey) and/or dantrolene (Sigma Chemical). Thirty minutes

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prior to the addition of SNP 50 or 500 μM, nimodipine 100 μM and 10 μM and/or dantrolene 100, 10, and 1 μM were added to the cultures and they were further incubated at 37 °C in humidified 95% air and 5% CO_2 for an additional 16 h.

Cultures were dyed with 1.5 ml of 0.4% trypan blue for 5—15 min, and neuronal cell death was assessed with the dye-exclusion test under an inverted light microscope by a scientist who was unaware of the content of the flasks.

The mean±S.E.M. scores of the neurons were calculated. The scores were translated to the percentage of the cell death population. They were analyzed statistically using analysis of variance (ANOVA) and then, using the independent-sample *t*-test, with $p<0.05$ considered indicative of significance.

RESULTS

All doses of the SNP were able to induce cell death with respect to control values ($p<0.001$ for all groups tested). Interestingly, a nonlinear dose–response curve was obtained for SNP-induced neurotoxicity (Fig. 1). Nimodipine and dantrolene failed to prevent neuronal cell death at both doses tested, either alone or in combination. Despite the fact that the most effective dose was found to be dantrolene 10 μM concentration for SNP 500 μM and 1 μM for SNP 50 μM, no statistically significant difference was seen for any dose tested. For further information, please refer to Figs. 2 and 3.

DISCUSSION

The effects of SNP on neuronal cells are still debatable. Most studies have found neurotoxic effects of it[12,13] while others found it to be a neuroprotective agent.[14] As a signalling molecule, NO was reported to induce an increase in blood perfusion in ischemic penumbra in the early stages of cerebral ischemia.[1] But a more recent study confirmed that the neuroprotective action of SNP was independent of an improvement in brain perfusion.[14] In our study, the neurotoxic effect of SNP was obvious at various doses. Some NO donors (*e.g.*, SNP) stimulate, while others (nitroglycerine, diethylamine/NO, NO in Ringer's solution) suppress hydroxyl radical generation *in vitro*.[15] In our study, SNP appeared cause to hydroxyl radical generation, an increase in the level of iron, lipid peroxidation, and neuronal death.

Nimodipine and dantrolene were found to be ineffective in preventing SNP-induced neurotoxicity in cerebellar granular cell culture of rat pups in the present study. As a dihydropyridine and voltage-gated calcium channel blocker, nimodipine had a dose-dependent neuroprotective effect in kainate-induced neurotoxicity in cerebellar neurons.[16] Another study confirmed that the Ca^{2+} channel blockers have neuroprotective potential against high potassium- or glutamate-induced-cell death in neuronal cell cultures.[17] Surprisingly, in the present study, we did not find the same effect of nimodipine against SNP-induced neurotoxicity.

The endoplasmic reticulum is the major intracellular Ca^{2+} storage site.[8] It plays several important physiological roles including the regulation of protein synthesis, folding, and targeting,[18] cellular calcium homeostasis,[19] and modulating cell death.[20] Oxidants, sulfhydryl active agents, and free radicals inhibit Ca^{2+}-ATPases and release Ca^{2+} from the endo-

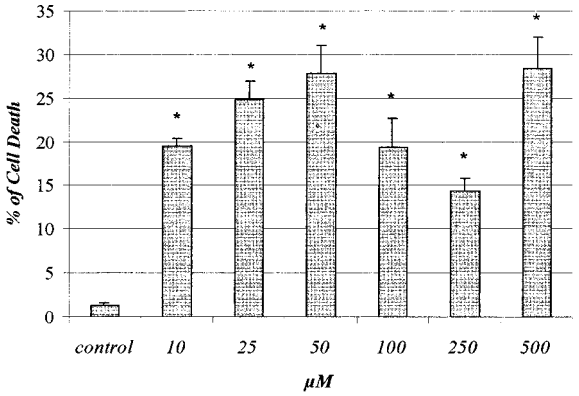


Fig. 1. The Effects of SNP on Cerebellar Neuronal Cell Cultures of Rat Pups

* $p<0.001$, with respect to controls, by ANOVA.

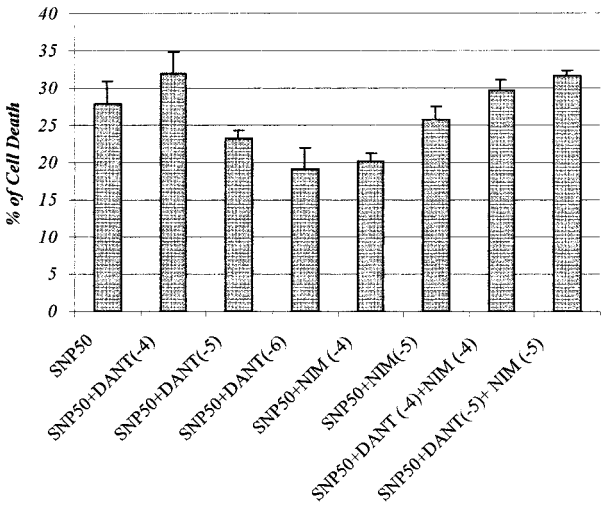


Fig. 2. The Effects of the Dantrolene and Nimodipine on SNP (50 μM)-Induced Neuronal Cell Death in Cerebellar Cultures of Rat Pups

(DANT(-4)=100 μM of dantrolene, DANT(-5)=10 μM of dantrolene, DANT(-6)=1 μM of dantrolene, NIM(-4)=100 μM of nimodipine, NIM(-5)=10 μM of nimodipine).

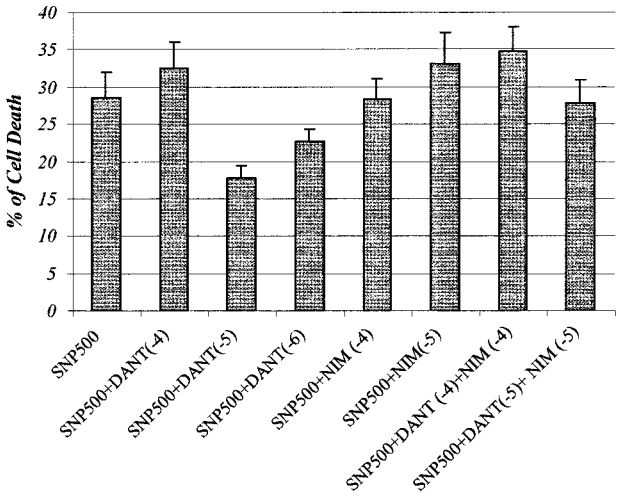


Fig. 3. The Effects of the Dantrolene and Nimodipine on SNP (500 μM)-Induced Neuronal Cell Death in Cerebellar Cultures of Rat Pups

(DANT(-4)=100 μM of dantrolene, DANT(-5)=10 μM of dantrolene, DANT(-6)=1 μM of dantrolene, NIM(-4)=100 μM of nimodipine, NIM(-5)=10 μM of nimodipine).

plasmic reticulum, suggesting that the endoplasmic reticulum Ca^{2+} could play a role in oxidant toxicity.[10] Dantrolene, as an endoplasmic reticulum Ca^{2+} release inhibitor, was reported to protect neural cells against death induced by glutamate.[21] Despite these results, we could not find a beneficial effect of dantrolene either alone or in combination with nimodipine on SNP-induced neurotoxicity. There are some controversial proposals for the role of intracellular calcium in oxidant-induced cell death. On the one hand, an increase in free calcium is thought to be nothing more than a late event associated with loss of membrane integrity and does not contribute appreciably to oxidant-induced cell death.[22] On the other hand, it has been proposed that an early increase in intracellular free calcium exacerbates oxidative stress, damages mitochondria, activates Ca^{2+}-dependent degradative enzymes, and disrupts the cytoskeleton, all of which play a central role in oxidant-induced cell death.[9] Our results are consistent with the first theory, and in SNP-induced neurotoxicity, calcium release probably occurs depending on the loss of membrane integrity of neurons. It was also reported that the blocking of intracellular Ca^{2+} release by dantrolene prevents cell death in cerebellar granular cell culture of rat pups, and furthermore this effect was found to be potentiated by nimodipine in glutamate-induced neurotoxicity.[23] It was reported that a low concentration of NO plays a protective role in glutamate neurotoxicity by closing the NMDA receptorgated ion channel, while elevated concentrations interacting with oxygen radicals, become toxic and mediate glutamate-induced neurotoxicity in cultured retinal neurons.[24] But, based on results of the present study, we speculate that SNP-induced neurotoxicity may act on some different mechanisms of glutamate receptors, L-type Ca^{2+} channels, and calcium currents, and endoplasmic reticulum calcium release. This effect was not dose dependent.

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