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ORIGINAL PAPER

Peroxynitrite is Involved in the Apoptotic Death of Cultured Cerebellar Granule Neurons Induced by Staurosporine, but not by Potassium Deprivation

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Abstract Nitric oxide (NO) regulates numerous physiological process and is the main source of reactive nitrogen species (RNS). NO promotes cell survival, but it also induces apoptotic death having been involved in the pathogenesis of several neurodegenerative diseases. NO and superoxide anion react to form peroxynitrite, which accounts for most of the deleterious effects of NO. The mechanisms by which these molecules regulate the apoptotic process are not well understood. In this study, we evaluated the role of NO and peroxynitrite in the apoptotic death of cultured cerebellar granule neurons (CGN), which are known to experience apoptosis by staurosporine (St) or potassium deprivation (K5). We found that CGN treated with the peroxynitrite catalyst, FeTTPs were completely rescued from St-induced death, but not from K5-induced death. On the other hand, the inhibition of the inducible nitric oxide synthase partially protected cell viability in CGN treated with K5, but not with St, while the inhibitor L-NAME further reduced the cell viability in St, but it did not affect K5. Finally, an inhibitor of the soluble guanylate cyclase (sGC) diminished the cell viability in K5, but not in St. Altogether, these results shows that NO promotes cell survival in K5 through sGC-cGMP and promotes cell death by other mechanisms, while in St NO promotes cell survival independently of cGMP and peroxynitrite results critical for St-induced death. Our results suggest that RNS are differentially handled by CGN during cell death depending on the death-inducing conditions.

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Introduction

Apoptosis is a type of programmed cell death that participates in the modelling of structures during development, the elimination of activated immune cells and transformed cells, among other processes. This is a fundamental and dynamic biological phenomenon characterized by DNA fragmentation, chromatin condensation, activation of caspases, translocation of phosphatidylserine and formation of apoptotic bodies [1–6]. It has been suggested that the production of reactive oxygen species (ROS) is critical for the initial phase of the apoptotic process. There are several reports showing that ROS are required for the progression of apoptotic death in neurons and glial cells [7–11].

On the other hand, reactive nitrogen species (RNS) have also been associated with the molecular mechanisms involved in the apoptotic cell death process [12, 13]. RNS are generated by nitric oxide synthases (NOS), which produce nitric oxide from L-arginine and oxygen. NO is a free radical and it is the main source for other RNS [14]. It is present in all tissues including the nervous system, where it serves major physiological roles such as synaptic plasticity, regulation of cerebral blood flow, modulation of neuroendocrine functions, among others [15].

Both, ROS and RNS are intimately related and may interact to induce the formation of reactive species that result critical for the apoptotic cell death. The imbalance of ROS levels increase the number of reactions between ROS and RNS, leading to nitrosative stress. The main reaction is the formation of peroxynitrite, which is produced by the

reaction between nitric oxide and superoxide anion [14]. Peroxynitrite is a highly reactive molecule that may induce alterations of macromolecules [16, 17] and induce apoptosis in a variety of cell types including neurons [12, 18]. In fact, some of the deleterious actions of nitric oxide in neurons can be attributed to the formation of peroxynitrite [19]. Furthermore, this molecule has been described to be critical for the mechanisms of neuronal injury in a variety of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [14, 20].

The actions of peroxynitrite are not limited to cell damage, low levels of peroxynitrite lead to the activation of Akt and by this way it may influence cell survival [21]. Both, nitric oxide and peroxynitrite are able to induce cell survival or cell death depending on the cell type and the cell context [22–25]. However, in contrast to ROS, there is little and controversial information about the possible role of RNS in the apoptotic cell death process and the mechanisms by which peroxynitrite induces apoptosis in neurons are unclear. For these reasons, in the present study, we assessed the participation of RNS during the apoptotic death of cerebellar granule neurons (CGN). These cells undergo apoptosis when they are treated with staurosporine (St), an inhibitor of protein kinases, or when they are transferred from a 25 mM [K+]-containing medium (K25) to a medium containing 5 mM [K+] (K5) [1–3, 26, 27]. The apoptotic process induced by these conditions is accompanied by the early production of ROS, suggesting that ROS could be critical in this process and raises the possibility that they could constitute an early signal in the apoptotic death of CGN [9, 28]. This possibility has been further strengthened by the observation that antioxidant treatment prevents cell death and that ROS can directly induce apoptotic death [10, 28–30].

In these model of apoptotic death, there is no substantial information about the role of nitric oxide, neither the possible involvement of peroxynitrite. To assess this, we induced apoptosis in CGN by St treatment and potassium deprivation and we examined the cell survival, apoptotic parameters and the levels of tyrosine nitration in the presence of a peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTPPS) and NOS inhibitors.

Materials and Methods

Materials

Fetal calf serum and penicillin/streptomycin were from GIBCO (Grand Island, NY, USA). Calcein-AM was from Invitrogen, Molecular Probes (Eugene, OR, USA). Poly-L-lysine (molecular weight_300,000), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), cytosine-D-arabino-furoanoside and staurosporine (St) were from Sigma (St Louis, MO, USA). FeTTPs was from Calbiochem, Merck. Others chemicals were of the purest grade available from regular commercial sources.

Cerebellar Granule Neurons Cultures

All animal used for the experimentation described in the present study were treated in accordance with the accepted standards of animals care and with the procedures approved by the local Committee of Research and Ethics of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. The protocol used followed the Guidelines for the Care and Use of Mammals in Neuroscience as well as guidelines released by the Mexican Institutes of Health Research and the National Institutes of Health guide for the care and use of Laboratory animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

CGN cultures were prepared as previously described [31]. Briefly, cell suspensions dissociated from 7-day-old rat cerebellum were plated at a density of 265×10^3 cells/cm^2 in plastic dishes or coverslips coated with poly-L-lysine (5 μg/ml) and were maintained for 7 days in vitro (DIV). Culture medium contained basal Eagle's medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 25 mM KCl, 50 U/ml penicillin and 50 mg/ml streptomycin. This medium is referred in text as K25 and is the control condition. The culture dishes were incubated at 37 °C in a humidified 5 % CO_2/95 % air atmosphere. To avoid non-neuronal cells, cytosine arabinoside (10 mM) was added 24 h after seeding. Cell death was induced by treating cells with staurosporine (St) (0.5 μM) or by transferring cells to a medium containing 5 mM KCl (K5). In some experiments, FeTTPs or NOS inhibitors were added simultaneously with K5 or St, and they were present until the end of the experiment.

Cell Viability

Cell viability was estimated by calcein and propidium iodide staining and by MTT transformation. Calcein enters to viable cells and emits green fluorescence when it is cleaved by esterases and cannot be longer permeable to cell membranes. Propidium iodide is not permeable to cell membranes and only death cells are stained. CGN were incubated with 2.5 μM calcein-AM and 2 μM and propidium iodide for 10 min at 37 °C, and then cells were photographed in a fluorescence microscope using filters with the following characteristics: excitation filter

wavelength/dichromatic mirror cut-on wavelength/barrier filter wavelengths of 450–490/500/515 and 510–560/565/590 nm for calcein and propidium iodide, respectively. Subsequent analysis involved the determination of calcein-positive cells from at least two different images for each condition. Results are expressed as the percentage of viable cells (calcein-positive cells) from the total number of cells (calcein-positive cells + propidium iodide-positive cells) evaluated per field.

CGN were incubated with MTT (0.5 mg/ml) for15 min in the culture medium at 37 °C. After incubation, the formazan crystals formed from MTT transformation were extracted with DMSO (100 %) and quantified spectroscopically at 560 nm. There was a good correspondence between MTT and calcein incorporation assays.

Nuclear Condensation

Cells were plated on coverslips and cultured for 7 days in vitro (DIV) and then treated to induce apoptosis. After 24 h of treatment, cells were fixed in 4 % paraformaldehyde for 20 min, and then incubated with Hoechst 332558 (0.01 %) in PBS for 5 min. Cells were examined with an epifluorescence microscope using a filter with following characteristics: excitation filter wavelength/dichromatic mirror cut-on wavelength/barrier filter wavelengths of 340/400/460 nm. Three different photographs taken at random were analyzed for each condition.

Caspase 3 Activity

After St treatment, CGN were washed with cold PBS and homogenized in lysis buffer [Tris–HCl (10 mM), NaH2PO4 (10 mM), NaCl (130 mM) and triton (1 %)]. Caspase 3 activity was determined by adding Ac-DEVD-MCA (Peptide Institute, Japan) diluted in an equal volume of reaction buffer [HEPES (40 mM), DTT (2 mM) and Ac-DEVD-MCA (20 μM)]. Fluorescence recording and the injection of the reaction buffer were performed with the microplate reader Synergy HT (BioTek Instruments, USA). Data were adjusted with protein concentration and normalized with respect to control.

Nitrosylation of Tyrosine Residues by Dot Blot Analysis

After 4, 8, 12 and 24 h of K5 or staurosporine (0.5 μM) treatment, CGN were washed with 37 °C PBS and homogenized in lysis buffer [25 mM Tris–HCl, 50 mM NaCl, 1 % (v/v) NP-40, 0.2 % (w/v) SDS, complete protease inhibitor cocktail], and the homogenates (30 μg of protein per lane) were spotted through circular templates directly onto the nitrocellulose membrane (BIO-RAD, Trans-Blot) using dot camera during 30 min. Then, the membranes were dry and blocked 1 h with 5 % nonfat dry milk in TBS buffer, and then incubated overnight at 4 °C with the primary antibody against Nitro-Tyrosine proteins and peptides containing nitro-tyrosine (Cell Signalling, Technology, USA, #9691).

Statistical Analysis

Statistical analysis was done by using SigmaPlot 12.1 software. Data are expressed as mean ± SEM. Pairwise comparison within multiples groups was done by analysis of variance (ANOVA) followed by the Tukey post hoc test; p values <0.05 were considered statistically significant.

Results

Peroxynitrite Mediates CGN Death Induced by St but not by Potassium Deprivation

In order to determine the role of RNS in the apoptotic death of CGN, firstly we assayed two apoptotic cell death models, St treatment and potassium deprivation. These conditions, induce in CGN the acquisition of different apoptotic characteristics such as caspase activation, DNA fragmentation, nuclear condensation and cytochrome c release, among others [27, 32]. These characteristics are accompanied by the early production of ROS, which is determinant for the completion of the apoptotic cell death process [9, 28]. Figure 1a, b show that the cell viability of CGN diminished in a time dependent manner under St treatment or when cells were transferred from depolarizing concentrations of potassium chloride (25 mM, referred as K25) to non-depolarizing concentrations of potassium chloride (5 mM, referred as K5). Under these conditions, the cell viability, seen by MTT transformation, diminished around 30–40 % at 12 h and 40–50 % at 24 h. After 24 and 48 h, the cell viability diminished around 10–20 %. Similar results were observed when the cell viability was evaluated by calcein staining (Fig. 1c) by showing that the cell viability diminished around 30–50 % at 24 h, corroborating that by 24 h, an important percentage of CGN had already died. The cell viability reduction could be observed by the morphological changes that CGN experienced during the apoptotic process. In control conditions, CGN had rounded cell bodies that tended to aggregate and from these aggregates numerous bundled processes were extended. When CGN are transferred to K5 or they are treated with St for 24 h, approximately half of the cell bodies shrank and the processes began to degenerate, which was more evident in St treated cells. After 48 h, almost all the cell bodies

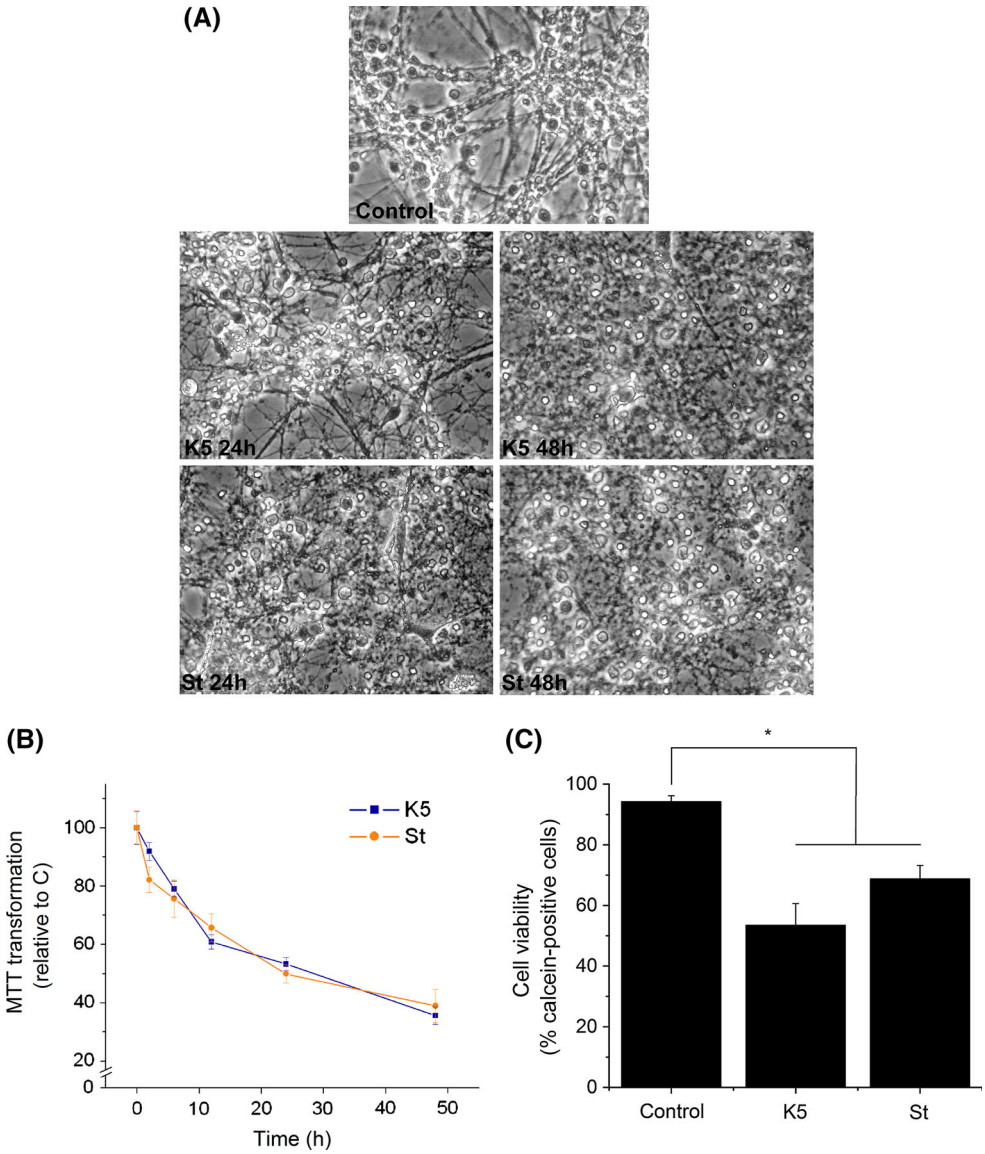

Fig. 1 Effect of K5 and staurosporine on the cell viability of CGN. **a** Representative micrographs of CGN of 7 DIV grown in depolarizing conditions (K25, Control) and transferred to non-depolarizing conditions (K5), or treated with St (0.5 μM) for the indicated times. **b** Cells were cultured for 7 DIV in (K25) and then transferred to K5 or treated with St (0.5 μM), and cell viability was evaluated by MTT transformation after 6, 12, 24 and 48 h or **c** after 24 h by calcein staining. Data are presented as mean ± SEM of 6 independent experiments (**b**) and 3 independent experiments (**c**). *Significantly different from control ($p < 0.05$, ANOVA)

were shrunken and the processes were completely damaged (Fig. 1a).

One of the most oxidative molecules produced by the reaction between RNS and ROS is peroxynitrite [33], which is known to induce apoptosis [12, 18]. For this reason, we studied whether this molecule was implicated in the apoptotic process induced by St and potassium deprivation. Figure 2a shows the normal morphology of CGN in phase contrast microcopy and the viable cells stained with calcein and non-viable cells labeled with propidium iodide staining. Under St treatment, the number of calcein-positive cells diminished and the correspondingly member of propidium iodide-positive cells increased. This effect was completely reverted by FeTTPs co-treatment. FeTTPs catalyzes the isomerization of peroxynitrite to nitrate [34]. Figure 2b shows that when CGN were treated with St for 24 h, the cell viability diminished around 45 %, which was completely rescued by FeTTPs. We tested different concentrations of FeTTPs, ranging from 1 to 100 μM, and only 100 μM FeTTPs completely rescued from cell death (not shown). Interestingly, when we added FeTTPs 12 h after the St treatment, we found that FeTTPs was still able to protect CGN from cell death, indicating that the effect of FeTTPs occurs after initiated the apoptotic process induced by St (Fig. 2c). In contrast, in the potassium deprivation model, FeTTPs (100 μM) induced the complete death of CGN and lower concentrations of FeTTPs, ranged from 0.1 to 10 μM, had no effect on cell viability (only FeTTPs 1 μM is shown), suggesting that peroxynitrite have little or no effect in the apoptotic process and showing a protective effect (Fig. 3a, b). This can be observed in the calcein staining assays where almost no calcein-positive cells were present under potassium deprivation and FeTTPs (100 μM) treatment (Fig. 3a).

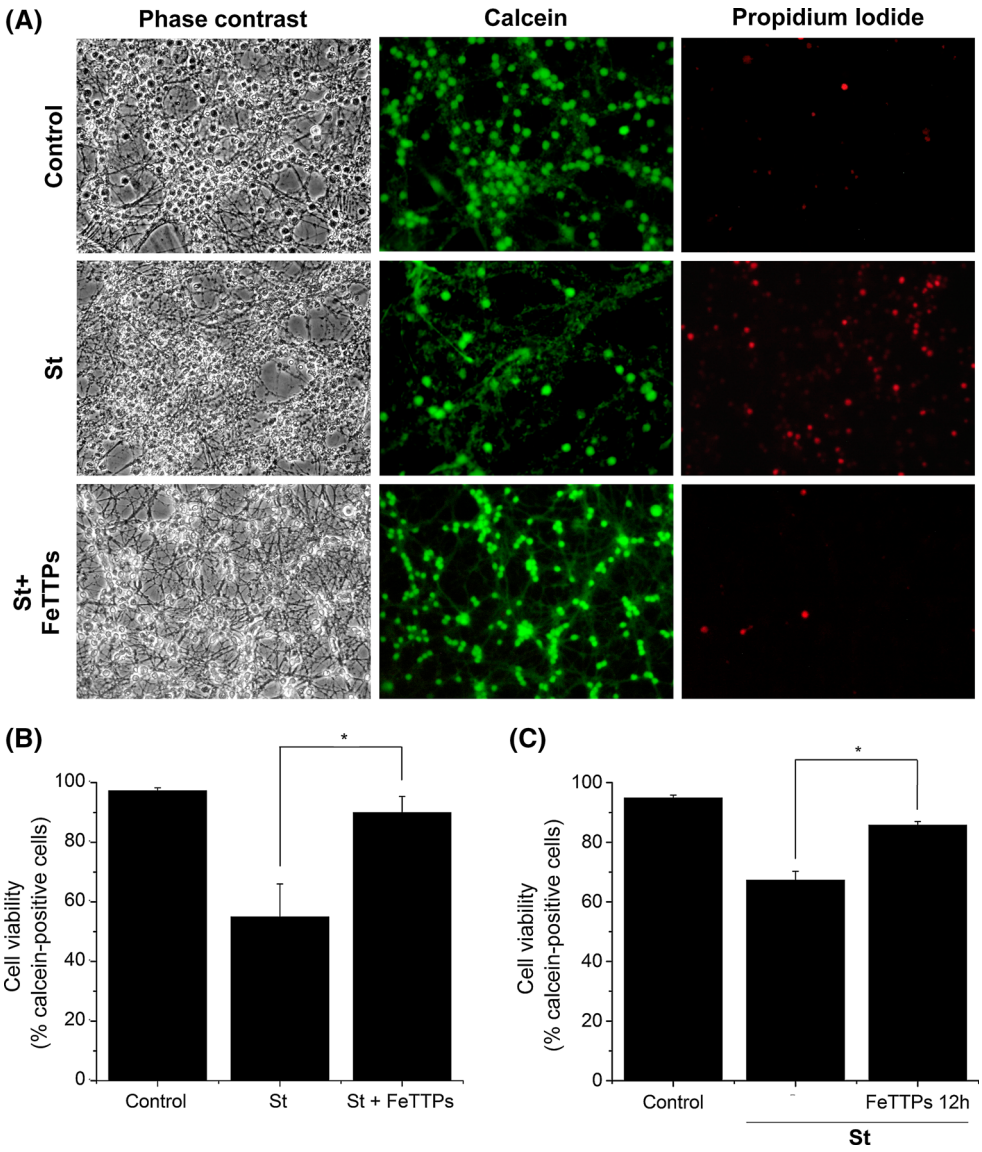

Fig. 2 Effect of FeTTPs on the CGN death induced by St. **a** Representative micrographs of CGN labelled with calcein and propidium iodide as detailed in Methods. CGN of 7 DIV grown in depolarizing conditions (K25, Control) treated with St (0.5 μM) for 24 h or St (0.5 μM) plus FeTTPs (100 μM) for 24 h. Panels are phase contrast images, calcein stained cells and propidium iodide stained cells. **b** Quantification of the cell viability of the CGN shown in (**a**). Data are presented as mean ± SEM of 3 independent experiments. *Significantly different from St ($p < 0.05$, ANOVA). **c** Quantification of the cell viability of CGN treated with St (0.05 μM) for 24 h plus FeTTPs (100 μM) for 12 h. Data are presented as mean ± SEM of 3 independent experiments. *Significantly different from St ($p < 0.05$, ANOVA)

Peroxynitrite Inhibits Caspase 3 Activity and Nuclear Condensation Under St Treatment

Two hallmark characteristics of the apoptotic process are nuclear condensation and the activation of the executioner caspase 3. Figure 4a shows that both, St treatment and potassium deprivation induced nuclear condensation of CGN after 24 h. Correspondently with the cell viability assays, FTTPs (100 μM) prevented the nuclear condensation only in St treatment and it reduced the total number of nuclei in potassium deprivation, but not the condensed nuclei. St increased by 60 % the number of condensed nuclei and FeTTPs significantly diminished this number (Fig. 4b). We also explored the possibility that the effect of FeTTPs in the apoptotic death induced by St occurs through the regulation of the caspase 3 activation. Figure 4c shows that St induced the activation of caspase 3 at early stages of the apoptotic process. After 2 h of treatment, the activity increased around threefold and continued increasing gradually, reaching a peak at 10 h. Caspase 3 activity was markedly reduced by FeTTPs, even when this compound were incubated after 2-4 h of St treatment, suggesting that the effect of FeTTPs is upstream of caspase 3 activation.

Tyrosine Nitration Induced by St and Potassium Deprivation is Reduced by FeTTPs

RNS induce different posttranslational modifications that includes S-nitrosylation, glutathionylation and tyrosine nitration, among which, tyrosine nitration is mainly mediated by peroxynitrite [14]. Here we found that both. St and

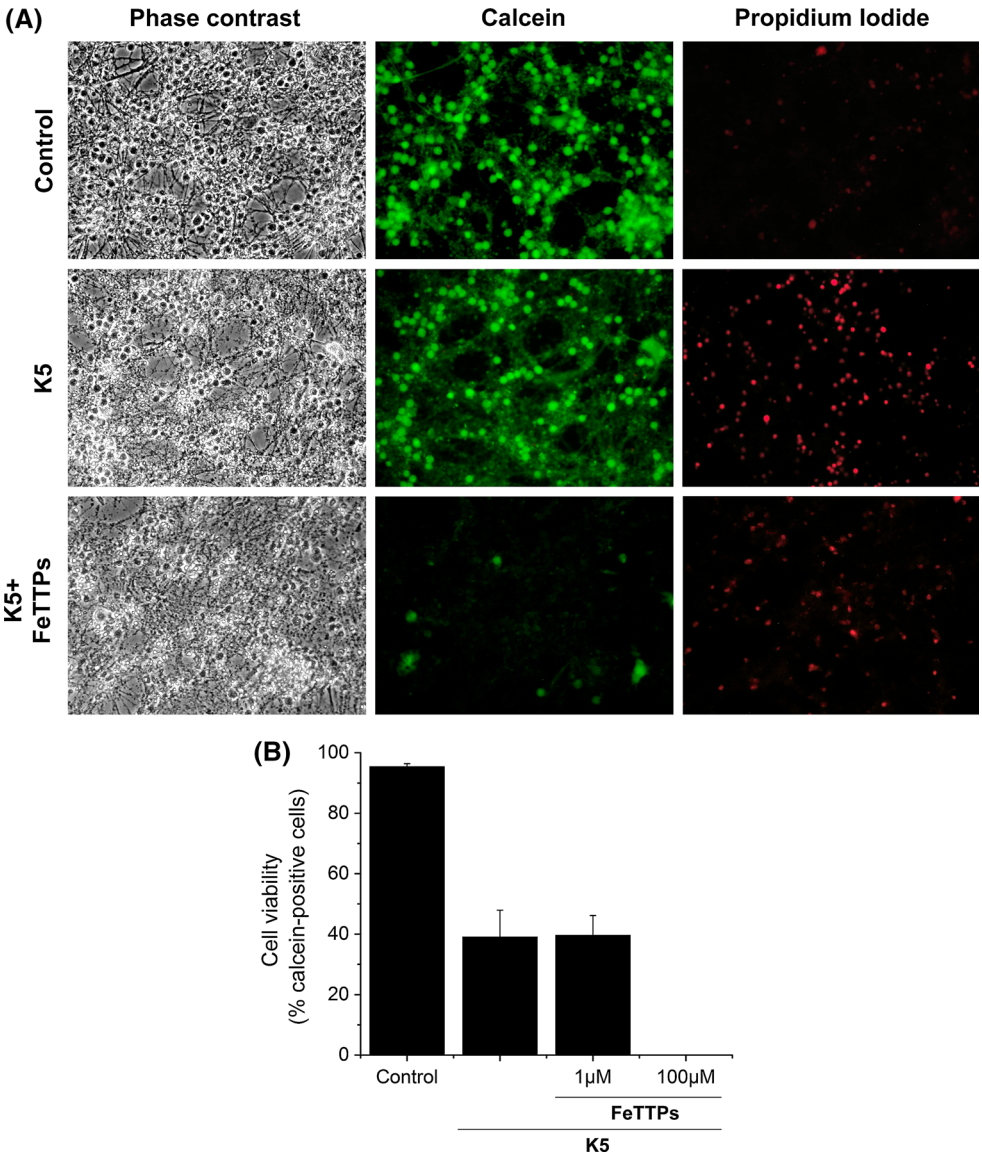

Fig. 3 Effect of FeTTPs on the CGN death induced by K5. **a** Representative micrographs of CGN labelled with calcein and propidium iodide as detailed in methods. CGN of 7 DIV were grown in depolarizing conditions (K25, Control) and transferred to non-depolarizing conditions (K5) for 24 h with or without FeTTPs (100 μM) for 24 h. Panels are phase contrast images, calcein stained cells and propidium iodide stained cells. **b** Quantification of the cell viability of CGN grown in K25 and transferred to K5 with or without different concentrations of FeTTPs (11 and 100 μM). Data are presented as mean ± SEM of 4 independent experiments. No significant differences were found between K5 and FeTTPs treatments (ANOVA)

potassium deprivation induced an increase of tyrosine nitration after 8 h, which was sustained until 24 h. After 4 h, the levels of tyrosine nitration in K5 and St were similar to control. Interestingly, under both conditions, FeTTPs (100 μM) markedly reduced the levels of tyrosine nitration (Fig. 5). These results demonstrated the production of peroxynitrite under these apoptotic conditions.

Nitric Oxide Inhibition Shows Differential Effects on the Death Induced by St and Potassium Deprivation

Finally, we tested the role of nitric oxide in the apoptotic death process, since this molecule might play a dual role in cell survival, On one hand, nitric oxide is the precursor of peroxynitrite and it is known that nitric oxide can cause neuronal death [35], which could be independent of peroxynitrite formation [36]. On the other hand, nitric oxide may have a neuroprotective effect through cGMP production in different models [37, 38]. In order to understand the role of nitric oxide in the apoptotic process induced by St and K5, we tested different NOS inhibitors and an inhibitor of the soluble guanylate cyclase in CGN survival under control and apoptotic conditions. Different concentrations of L-NAME (100, 200 and 500 μM) did not alter the overall appearance of the cell culture (Fig. 6a), or reduced the cell viability of cells (Fig. 6b) or protected against the cell death induced by potassium deprivation or St (Fig. 6c (only L-NAME 500 μM is shown). Even higher concentrations of L-NAME (1–10 mM) did not protect against the cell death induced by potassium deprivation and St (Table 1). Only 10 mM L-NAME reduced cell viability in

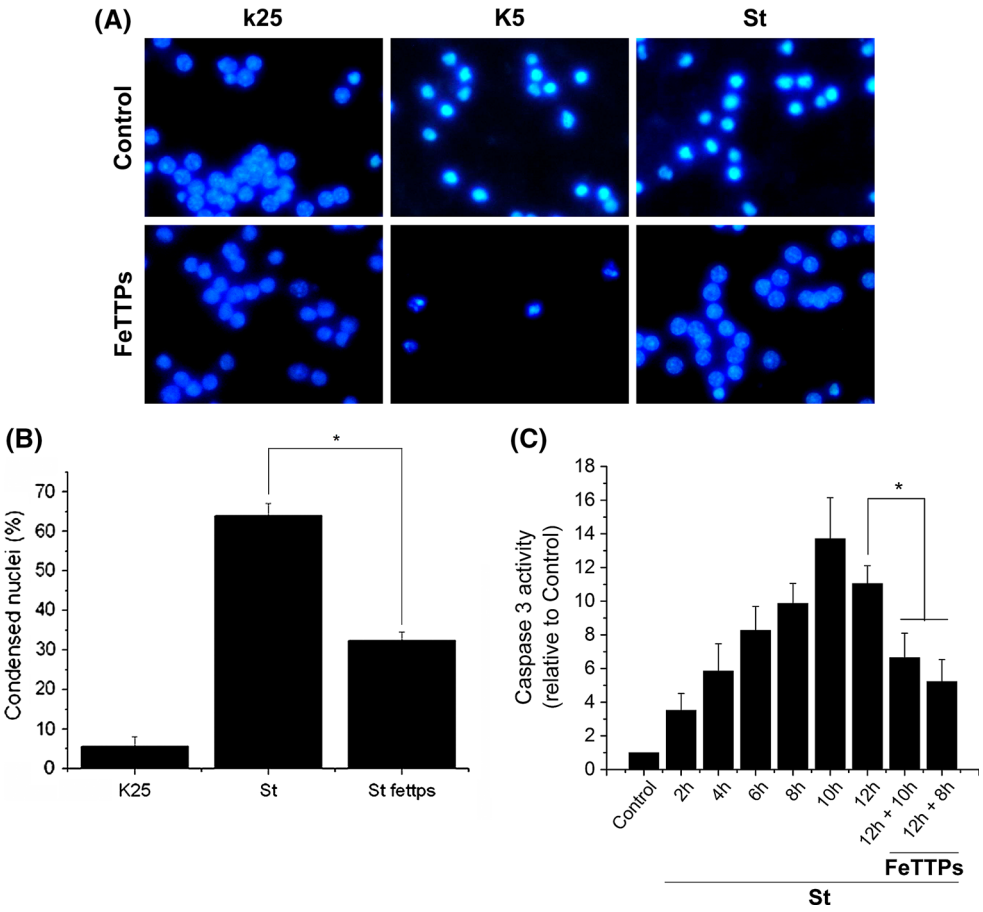

Fig. 4 Effect of FeTTPs on the nuclear condensation induced by K5 and St and in the caspase 3 activity induced by St. **a** Representative micrographs of CGN stained with Hoechst 33258 as detailed in Methods. CGN of 7 DIV were grown in depolarizing conditions (K25) and transferred to non-depolarizing conditions (K5) or treated with St for 24 h with or without FeTTPs (100 μM). **b** Quantification of the St treatment shown in (**a**). Data are presented as mean ± SEM of 3 independent experiments. *Significantly different from St ($p < 0.05$, ANOVA). **c** Caspase 3 activity was evaluated as detailed in methods. CGN of 7 DIV were treated with St (0.5 μM) for 2, 4, 6, 8, 10 and 12 h and St (0.5 μM) for 12 h plus FeTTPs (100 μM) for 10 and 8 h and caspase 3 was evaluated. Date are presented as mean ± SEM of 4 independent experiments. *Significantly different from St 12 h ($p < 0.05$, ANOVA)

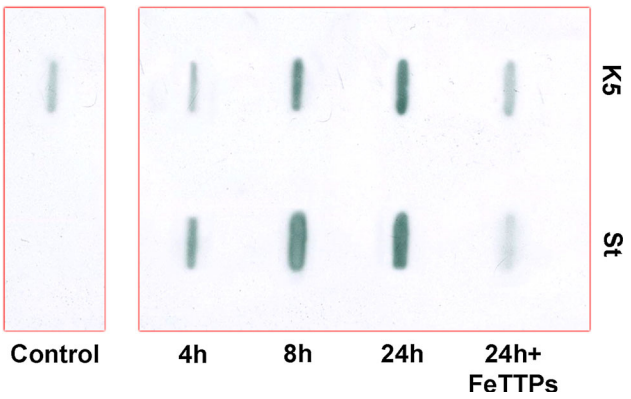

Fig. 5 Effect of FeTTPs on tyrosine nitration induced by K5 and St. Representative blot of tyrosine nitrated proteins extracted from CGN of 7 DIV grown in depolarizing conditions (K25), or transferred to non-depolarizing conditions (K5) or treated with St (0.5 μM) for the indicated times. Cells were cultured for 7 DIV in (K25) and then transferred to K5 or treated with St (0.5 μM) for 4, 8 and 24 h with or without FeTTPs (100 μM) for 24 h. Cell lysates (30 μg of protein per line) were directly transferred to a nitrocellulose membrane by using a dot camera. Three independent experiments were performed

control conditions and with St treatment (Table 1). L-NAME is a competitive inhibitor of endothelial NOS (IC_{50} 10 μM), neuronal NOS (IC_{50} 60 μM) and inducible NOS (IC_{50} 11.5 μM), once this molecule is hydrolyzed by esterases inside the cells, it is converted into L-NNA and interact non-covanlently with all NOS. The union of this molecule with endothelial NOS (eNOS) and neuronal NOS (nNOS) is more stable than the union with the inducible NOS (iNOS) [39], conferring certain selectivity of eNOS and nNOS over iNOS, thereby the observed effects seen in St and K5 are probably are related to the activation of the iNOS.

To evaluate this possibility, we tested two non-selective NOS inhibitors, 2-ethyl-2-thiopseudourea hydrobromide (EPTU, IC_{50} values for eNOS, nNOS and iNOS of 0.37, 0.25 and 0.13 μM, respectively) and 7-nitroindazole (7-NI, IC_{50} values for eNOS, nNOS and iNOS of 0.78, 0.71 and 5.8 μM, respectively). Table 1 shows that the cell viability measured by MTT was slightly reduced by EPTU and 7-NI in control cells at the higher concentrations used. In K5 treated cells, EPTU, but not 7-NI, increased the cell viability. No significant changes were observed in the group treated with St and EPTU or 7-NI. These results suggest that nitric oxide is required for cell survival in control conditions and probably under St treatment. On the other hand, nitric oxide might be necessary for the process of cell

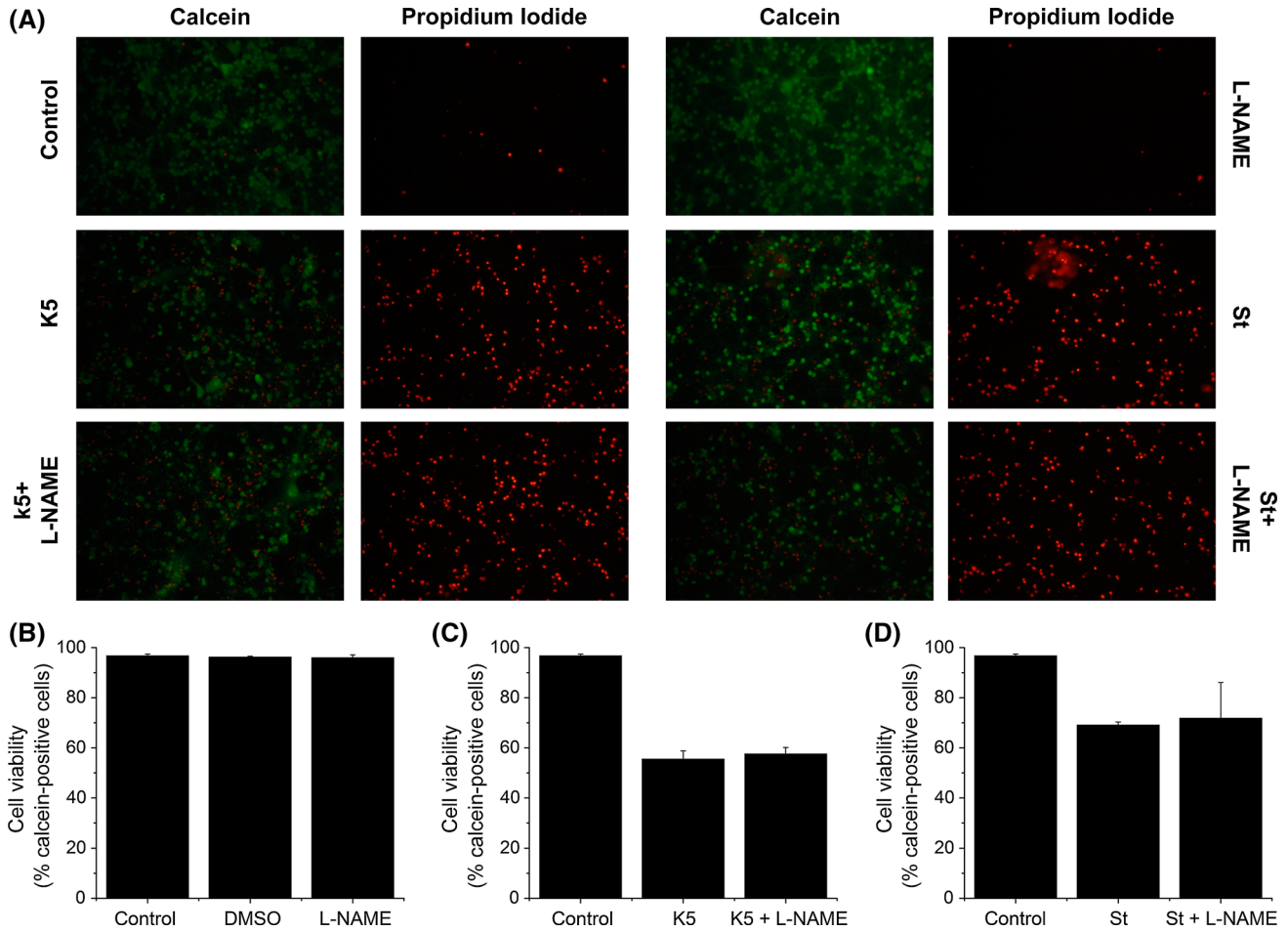

Fig. 6 Effect of L-NAME on the cell death induced by K5 and St. **a** Representative micrographs of CGN labelled with calcein and propidium iodide as detailed in Methods. CGN of 7 DIV were grown in depolarizing conditions (K25) and transferred to non-depolarizing conditions (K5) for 24 h or treated with St (100 μM) plus L-NAME (500 μM) for 24 h. **b** CGN of 7 DIV were grown in K25 and treated with DMSO (0.8 % v/v) for 24 h, or L-NAME (500 μM). Data are presented as mean ± SEM of 5 independent experiments. No significant differences were found between the treatments (ANOVA). **c** Cell viability was measured by calcein staining in CGN of 7 DIV grown in depolarizing conditions (K25) and transferred to non-depolarizing conditions (K5) for 24 h and treated with L-NAME (500 μM) for 24 h or **d** treated with St (0.5 μM) for 24 h and L-NAME (500 μM) for 24 h. Data are presented as mean ± SEM of 5 (**c**) and 4 (**d**) independent experiments. No significant differences were found between the treatments (ANOVA)

death induced by K5. We found a tendency of the inhibitors EPTU and L-NAME (10 mM) to reduce the cell viability in St treatment, which suggest that nitric oxide might have a pro-survival effect under this condition.

The main signaling pathway activated by nitric oxide involves soluble guanylate cyclases (sGC) that seems to be involved in the neuroprotective effects exerted by nitric oxide [37, 38]. To clarify the possibility that some of the above described actions of NO are mediated by sGC, we tested the effect of ODQ, a specific inhibitor of sGC, on the cell viability measured as MTT transformation. Table 1 shows that ODQ treatment reduced the cell viability only under K5 conditions, suggesting a possible protective action of the nitric oxide-cGMP pathway in the K5-induced cell. Similar results were obtained when viability was evaluated with calcein and propidium iodide staining (data not shown).

Discussion

In the present study we found that the neuronal apoptotic death may show different molecular mechanisms depending on the initial mechanism for the induction of apoptotic cell death. Particularly, we showed that reactive species play a differential role when cells are treated with two different apoptotic conditions in the same cell type. In previous studies we and others showed that ROS play a critical role acting as signals in apoptotic death of CGN induced by both K5 and St [7, 9, 30, 40]. Here, we further

Table 1 Effect of soluble guanylyl cyclase and NOS inhibitors on cell viability of CGN treated with apoptotic conditions

Treatment	Cell viability (%)		
	K25	K5	St
None	100	60.3 ± 2.6*	55.3 ± 8.7*
EPTU 100 μM	100.8 ± 5.4	74.7 ± 5.4*,+	58.5 ± 6.3*
EPTU 700 μM	90.0 ± 1.2*	77.4 ± 3.0*,+	40.9 ± 4.0*
None	100	60.3 ± 5.4*	40.5 ± 6.3*
L-NAME 1 mM	100.8 ± 0.9	67.5 ± 5.4*	54.9 ± 7.2*
L-NAME 10 mM	86.4 ± 6.3*	71.1 ± 2.7*	27.0 ± 1.8*,+
None	100	64.8 ± 8.1*	59.4 ± 2.7*
7-NI 10 μM	91.8 ± 2.7*	63.9 ± 4.0*	62.5 ± 1.8*
7-NI 100 μM	78.3 ± 2.7*	48.6 ± 2.7*	51.3 ± 2.7*
None	100	59.1 ± 2.6*	51.3 ± 8.7*
ODQ 10 μM	98.3 ± 4.3	41.7 ± 2.1*,+	45.2 ± 8.7*
ODQ 100 μM	104.4 ± 3.5	45.2 ± 6.7*	54.9 ± 6.7*

Cells were treated during 24 h with different concentrations of EPTU, L-NAME, 7-NI and ODQ with and without apoptotic stimulus (K5, St) and were assayed for MTT reduction as detailed in Methods. In ODQ and 7-NI assays, the vehicle (DMSO) was added to the control. Results are expressed as the percentage of control (K25) for each group of data. Data are presented as mean ± SEM of 6 independent experiments. For each group, * significantly different from its K25 condition ($p < 0.05$, ANOVA); $^{+}$ significantly different from K5 or St alone

suggest that peroxynitrite is necessary for the progression of apoptosis induced by St, and that in the case of K5-induced death it might act as an anti-apoptotic molecule.

These results are in line with some of our previous studies in which we found differences in the signaling pathways involved in the apoptotic process induced by these two conditions. For example, we demonstrated that JNK and p38 pathways mediate the apoptotic death of CGN induced by K5, but cell death induced by St is mediated only by p38 [7, 8]. Also, in contrast to K5-induced cell death, the ROS generation in St-induced death is downstream of p38 activation. Our present results showed a marked neuroprotective action of the peroxynitrite decomposition catalysts FeTTPs in CGN treated with St. These results are in agreement with the observed effect of FeTTPs on the apoptotic parameters evaluated, nuclear condensation and caspase 3 activity.

In contrast to St, FeTTPs did not modify any of the evaluated apoptotic parameters under K5 conditions. Moreover, this compound markedly increased the cell death induced by K5 suggesting a possible protective action of peroxynitrite under these conditions. The idea that peroxynitrite may be beneficial has previously been proposed in other models of brain injury induced by NMDA [41] and excitotoxicity mediated by glutamate [42], as well as in a model of myocardial ischemic post-conditioning followed by episodes of ischemia–reperfusion injury [25].

An interesting result from our study was the observed protective effect of FeTTPs when it was administered to CGN after long periods of St treatment, which corresponds with a time when caspase 3 is already active. A similar delayed neuroprotective action of FeTTPs has been shown in a model of cerebral ischemia [43]. One possibility to explain this delayed action is that peroxynitrite could be acting upstream or directly on active caspase 3 and therefore reducing the effects of the apoptotic process. It is well established that S-nitrosylation and denitrosylation of proteins can be an important mechanism to modify the function of several proteins [44, 45]. Caspase 3 activity can be regulated by nitrosylation [46], tyrosine nitration and glutathionylation [47], which are events mediated by RNS [14]. It is known that thioredoxin induces transnitrosylation of Cys_{163} of caspase 3 and by this way it inhibits caspase activity [48]. Peroxynitrite can also regulate caspase 3 activity in a different way. For example, peroxynitrite induces caspase 3 activation in a model of spinal cord injury [49] and in a model of cerebral ischemia [50], but it also induces its inactivation by tyrosine nitration of caspase 3 in a model of traumatic brain injury [51]. These studies show that peroxynitrite may exert different actions on caspase 3 activation through posttranslational modifications.

In support of this idea, it has been shown in skeletal muscle that low exogenous levels of peroxynitrite (0.1 mM) inactivate the sarco/endoplasmic reticulum calcium (Ca2+) ATPase (SERCA) by inducing nitrosylation of the Cys_{349}, probably mediated by a direct interaction of peroxynitrite on the molecule or by transnitrosylation through the initial modification of other cysteine residues [52]. In contrast, physiological levels of peroxynitrite in smooth muscle increase the activity of SERCA by reversible glutathionylation of this protein [53]. These results demonstrate that peroxynitrite may mediate tyrosine nitration, as well as nitrosylation and glutathionylation of cysteine residues in a variety of proteins, but more importantly, these studies show that the levels of peroxynitrite may determine its role in the activity/inactivity state of the protein. Based on these evidences and due to the fact that FeTTPs reduced the caspase 3 activity at a time when caspase 3 was highly active and that peroxynitrite catalysis exacerbates the cell death induced by K5, we speculate that peroxynitrite could regulate caspase 3 in these models.

It is interesting that both, K5 and St induced a clear increase in the levels of tyrosine nitrated proteins, which was markedly reduced by FeTTPs in both cases. As discussed above, FeTTPs only inhibited the apoptotic process

induced by St, but not by K5. These results firstly suggest that both St and K5 induce peroxynitrite formation; and secondly that peroxynitrite may have differential roles. In the case of St, it is possible that peroxynitrite could induce apoptotic death through the tyrosine nitration, nitrosylation or glutathionylation of proteins. In fact, peroxynitrite induce tyrosine nitration of cytochrome c [54], cytochrome c release [55] and the activation of caspases 8 and 9 [55], which probably mediates caspase 3 activation. In the case of K5, peroxynitrite may induce tyrosine nitration or cysteine nitrosylation of caspase 3, therefore promoting an anti-apoptotic effect. Further experiments will be required to elucidate these possibilities.

It has been reported that nitric oxide may induce cell death by different mechanisms. Nitric oxide can produce damage either by a direct action on proteins or through peroxynitrite. We therefore investigated the possible role of nitric oxide in the process of cell death induced by both, K5 and St. Our results showed that all NOS inhibitors failed to rescue CGN from cell death induced by St, suggesting that nitric oxide does not promote cell death, which could be apparently contradictory to the results on peroxynitrite requirement for St-induced death, since peroxynitrite is a product of nitric oxide [33]. This can be explained by a possible generation of nitric oxide mediated by S-nitrosothiols that can act as nitric oxide reservoirs [56]. On the other hand, L-NAME further increased the cell death induced by St. Interestingly, all NOS inhibitors tested reduced the cell viability of CGN maintained in basal conditions. These results suggest that under these conditions nitric oxide could exert a neuroprotective action. This possibility has been extensively documented in several preparations, including developing CGN [22, 57, 58] and mature CGN [38, 59–61].

In contrast to St, the inhibition of NOS by EPTU, significantly reduced the cell death induced by K5, which support the idea that nitric oxide could be necessary for the apoptotic death induced by this condition. Thus, as previously mentioned, nitric oxide could play a dual action in our preparation. On one side, it may act as a physiological and neuroprotective agent probably by activating the cGMP/PKG cascade [38, 59] or through some other no-cGMP-dependent function of nitric oxide [62, 63]. On the other hand, nitric oxide and its derivative peroxynitrite could directly interact with several proteins, which could lead to alterations in their function, resulting in cell death, as it could be the case for the K5-induced cell death mediated by nitric oxide or the St-induced death mediated by peroxynitrite.

Finally, we explored the possibility that cGMP/PKG cascade could mediate the protective effect of nitric oxide in St-induced death. Our results using ODQ showed no changes in the cell viability, suggesting that the neuroprotective action of nitric oxide in this condition is not mediated by this cascade. However, ODQ treatment diminished the cell viability in K5, suggesting that the cGMP/PKG cascade promotes cell survival in this model. These results show a dual role of nitric oxide in the same model. On one hand, nitric oxide favor K5-induced death in a mechanism independent of the cGMP/PKG cascade. On the other hand, nitric oxide promotes cell survival in K5-induced death, which depends on the cGMP/PKG cascade.

In conclusion, these evidences show a differential regulation of the apoptotic pathway by RNS in the same cell type under two different apoptotic conditions. This suggests that the inductor of apoptotic death determines the initial mechanism involving reactive species. Our results suggest a limited participation of nitric oxide in the apoptotic death induced by St and a partial contribution in the K5-induced death of CGN. In contrast to K5, peroxynitrite seems to play a critical role in the death process induced by staurosporine in cerebellar granule neurons.

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