INVOLVEMENT OF FREE RADICALS IN SIGNALLING OF LOW-POTASSIUM INDUCED APOPTOSIS IN CULTURED CEREBELLAR GRANULE CELLS

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During the development of the nervous system approximately half of all neurons die by a process termed programmed cell death, PCD (Oppenheim, 1991). It is thought that there is a competition between neurons for trophic factors produced and released by target cells innervated by these neurons. Neurons that receive trophic factors go on maturation and those that do not receive it die. Thus, the competition for trophic factors could match neuronal number to target size. Cells dying by PCD show a set of morphological and biochemical changes, such as cell shrinkage, chromatin condensation and fragmentation. This is also called apoptosis to distinguish it from the accidental cell death or necrosis (Wyllie and cols., 1980).

It has been shown that many antioxidants protect cells from apoptosis. The finding that Bcl-2, the first mammalian PCD regulator discovered, has antioxidant properties (Hockenbery and cols., 1993) have led to suggest that reactive oxygen species (ROS) could be essential mediators in the signalling of apoptosis. Moreover, it has been demonstrated that metal ions, specially copper that generates free radicals in Fenton reaction, induce apoptosis from diverse stimuli in rat thymocytes (Wolfe and cols., 1994). However, it remains to be established the biochemical processes in which ROS are involved.

Primary cultures of cerebellar granule neurons are useful model systems for studying the biochemical mechanisms underlying apoptosis. In culture, the survival of these neurons is greatly enhanced by high extracellular potassium concentrations (Gallo and cols., 1987). When granule neurons in a high potassium medium are switched to a more physiological medium (5 mM KCl) they die by apoptosis. Increased $[K^+]_o$ depolarizes cells and seems to promote *in vitro* survival by mimicking effects of naturally occurring electrical activity, but the molecular mechanisms by which increased $[K^+]_o$ prevents apoptosis are not known. In this study we have investigated the effects of several antioxidant agents on neuronal death induced by low extracellular potassium concentrations and the possible role of free radicals in signalling apoptosis.

Methods

Neuronal cultures. Cerebellar granule neurons were prepared from cerebella of 7 days old Wistar rats. The cells were plated on 35-mm culture Corning dishes precoated with 10 mg/l poly-L-lysine at a density of 2.5×10^6 cells/dish in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (Sigma), 0.1 mU/ml insulin, 7 μM p-aminobenzoic acid, 0.1 mg/ml gentamicin, 0.1 mg/ml pyruvate and 25 mM KCl. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 48 h the medium was renewed to add 10 μM cytosine arabinofuranoside in order to prevent replication of non-neuronal cells.

Treatments of cultures. All experiments were performed in the fifth day of culture. At 5 DIV the medium was replaced with serum free DME/F12 (1:1) medium supplemented with 0.1 mg/ml transferrin, 20 nM progesterone, 50 U/ml penicillin, 37 U/ml streptomycin, 0.1 mg/ml pyruvate, 135 mU/ml insulin and either 5 or 25 mM KCl.

Cell viability. Neuronal viability was assesed by the 3-(4,5-dimethyl-thiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test. Viable cells reduce MTT to formazan which can be determined spectrophotometrically. In brief, the culture medium was replaced with 2 ml of Locke's buffer and incubated for 15 min with 0.3 mg MTT. Thereafter, formazan was dissolved in 1 ml DMSO and measured at 490 nm. Additionally, the extent of cell killing was assesed by trypan blue inclusion.

DNA fragmentation analysis. Cells were detached from the culture dish and then lysed in 5 mM Tris buffer (pH 7.4), 20 mM EDTA (TE buffer), 0.5% Triton X-100 for 1 h at 4°C. Nuclei were pelleted by centrifugation for 15 min at 10000 g. The supernatant was treated with 0.1 mg/ml proteinase K for 12-18 h. Fragmented DNA was extracted with phenol/chloroform and then precipitated with ethanol. DNA was then resuspended in 10-15 μl of TE buffer and treated with 1 μg/ml RNase. Fragmented DNA was determined fluorometrically by using Hoescht 33258 and DNA from calf thymus as standard. The rest was electrophoresed through a 1.2% agarose gel and visualized by UV ilumination after staining with ethidium bromide.

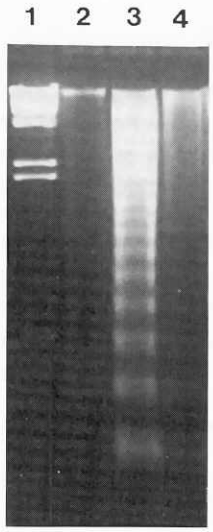

Figure 1. Inhibition of internucleosomal cleavage by SOD. Cells were incubated for 24 h in DMEM/F12 supplemented with 25 mM KCl (lane 2), 5 mM KCl (lane 3) or 5 mM KCl plus 24000 U/ml (lane 4). Following treatment, DNA was subjected to electrophoresis on a 1.2% agarose gel. Standard markers (lambda phage treated with *Hind* III) are shown in the first lane.

Results

The presence of 25 mM KCl and 10% FBS in the DME medium allowed cultured neurons mature after 5 DIV. In the fifth day of culture and after replacing the medium to the synthetic medium (DME/F12) plus 25 mM KCl only 2% of cell death was detected after 24 h. The effect of 25 mM KCl on cell viability was extended for 20 DIV supplementing cells with 5 μl glucose 20% every 3 days. However, when this medium was replaced to a low-potassium medium (5 mM) the ladder pattern of DNA fragmentation was observed when DNA was electrophoresed (see Figure 1, lane 3). This pattern of fragmentation is due to the activation of endonucleases which break DNA, generating internucleosomal size fragments, and can be considered as the best marker of apoptosis. The quantity of the fragmented DNA shown that the maximal effect was found after 24 h. When measured with the MTT reduction assay, a loss of 35% viability was detected accompanying this fragmentation (Figure 2 and 3).

It has been extensively documented that several metal ions participate in biological redox reactions in which free radicals are released. In one of these reactions the metal cation, usually ferrous or cuprous ions, combine with hydrogen peroxide giving ferric, or cupric ions plus hydroxyl radical and the hydroxide ion as products of the reaction. These metal ions are essential for the activity of several enzymes that bind to DNA during their catalytic cycle. These free radicals could damage DNA, thus triggering apoptosis. It has been reported that dexamethasone and etoposide induce apoptosis in thymocytes through a mechanism in which copper acts as a mediator. Due to this reason we have checked the possibility that free radicals released in these type of reactions could be acting in our cultures.

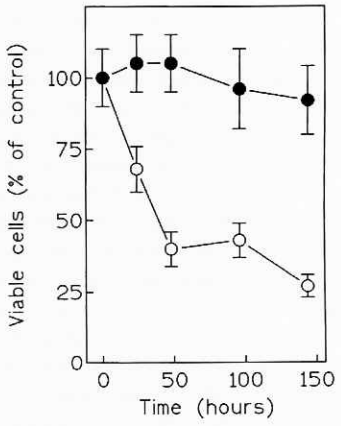

Figure 2. Time course of neuronal death caused by low K^+. Five days after plating, neurons were switched to serum-free medium containing 5 mM (open circles) or 25 mM KCl (filled circles). At the indicated times after the change of medium, the estimation of viable neurons was performed by the MTT reduction assay. Results are expressed as percent of the values obtained at 5 DIV.

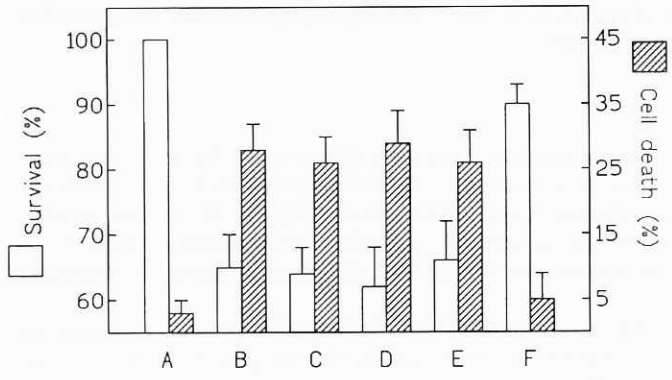

Figure 3. Effects of SOD and metal chelators on low-potassium induced apoptosis. Cells were incubated in 25 mM KCl for 24 h (A), or in 5 mM KCl (B to F) supplemented with 100 nM 1,10-phenanthroline (C), 5 nM neocuproine (D), 50 nM desferoxiamine (E), and 24000 U/ml SOD (F). Survival (open bars) and cell death (filled bars) were assesed by MTT reduction assay and trypan blue inclusion, respectively. The results represent the mean ± S.D. of three separated preparations.

For this purpose metal chelators, such as desferoxiamine (which complexes iron), neocuproine (specific for copper) and 1,10-phenanthroline (which binds both iron and copper), were added separately to cultures in the synthetic medium supplemented with 5 mM KCl. Non-toxic concentrations of these chelators resulted ineffective on both survival (see figure 3) or DNA fragmentation. These results strongly suggest that free radicals released in Fenton reaction are not involved in the signalling of low-potassium induced apoptosis in cerebellar granule neurons.

Another common free radical in aerobic cells is superoxide anion ($\cdot O_2^-$). To check wether $\cdot O_2^-$ is promoting apoptosis in these cells we added superoxide dismutase (SOD) to the external medium. Interestingly, SOD totally prevented DNA fragmentation (see figure 1, lane 4) and enhanced the cell viability to a level similar to that found in 25 mM KCl after 24 h. The maximal effect was found with 24000 U/ml SOD. The observation that SOD but not catalase prevent apoptosis excluded the possible role of H_2O_2 in this process. The strong effect of SOD suggests that $\cdot O_2^-$ could be an essential ROS mediating apoptosis upon lowering external potassium concentration. Moreover, the fact that only external SOD activity, and not internal SOD activity, was required to blockade DNA fragmentation, suggests that $\cdot O_2^-$ is released near the plasma membrane as one of the first steps in the signal transduction cascade. In conclusion, $\cdot O_2^-$ is involved in the triggering of apoptosis in cerebellar granule cells.

References

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