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Role of Oxidative Stress in the Apoptotic Cell Death of Cultured Cerebellar Granule Neurons

Antonio Valencia and Julio Morán*
Department of Neurosciences, Institute of Cell Physiology, National University of Mexico, Mexico City, Mexico

When cultured cerebellar granule neurons (CGN) are transferred from 25 mM KCl (K25) to 5 mM KCl (K5) caspase-3 and caspase-8, but not caspase-1 or caspase-9,activities are induced and cells die apoptotically. CGN death was triggered by a $[Ca^{2+}]_i$ modification when $[Ca^{2+}]_i$ was reduced from 300 nM to 50 nM in a K5 medium. The $[Ca^{2+}]_i$ changes were followed by an increase in ROS levels. The generation of both cytosolic and mitochondrial reactive oxygen species (ROS) occurred at three different times, 10 min, 30 min and 3–4 hr but only those ROS produced after 3–4 hr are involved in the process of cell death. When CGN cultured in a K5 medium are treated with different antioxidants like scavengers of ROS (mannitol, DMSO) or antioxidant enzymes (superoxide dismutase and catalase) phosphatidylserine translocation, caspase activity, chromatin condensation and cell death is markedly diminished. The protective effect of antioxidants is not mediated through a modification in $[Ca^{2+}]_i$. Caspase activation, PS translocation and chromatin condensation were downstream of ROS production. In contrast to H_2O_2, ROS produced by a xanthine/xanthine oxidase system in CGN cultured in K25 were able to directly induce caspase-3 activation and death that resulted sensitive to z-VAD, a caspase inhibitor. These findings indicate that a reduction in $[Ca^{2+}]_i$ triggers CGN death by inducing a generation of ROS after 3–4 hr, which could play a critical role in the initial phases of the apoptotic process including PS translocation, chromatin condensation and the activation of initiator and executor caspases. J. Neurosci. Res. 64: 284–297, 2001.

Key words: apoptosis; caspases; cerebellar granule neurons; ROS, antioxidants; calcium

Programmed neuronal death (PND) is a normal event during the development of the nervous system that significantly contributes to the shaping of brain (Oppenheim, 1990). It has been shown that PND is precisely controlled by extracellular influences, and it has been characterized as an active process regulated by an internal program, which can be blocked by protein and mRNA synthesis inhibition (Oppenheim, 1990). PND can be recognized from other types of cell death by some morphological and biochemical parameters that include nuclear condensation, vacuolization of cytoplasm, formation of phagosomes, loss of mitochondrial function, DNA fragmentation into nucleosomal-size fragments, activation of specific enzymes and proteases, etc. (Martin and Green, 1995; Cohen, 1997; Nardi et al., 1997; Ni et al., 1997; Morán et al., 1999). Several studies have been directed to understand the mechanisms involved in the process of PND. A large variety of molecules are involved in this process by acting as antiapoptotic or proapoptotic elements like the caspase family that plays a key role in PND. Particularly, caspase-3, which is a protease that participates in apoptosis in many tissues (Fernandes-Alnemri et al., 1994; Martin and Green, 1995; Tewari et al., 1995; Posmantur et al., 1997), and is constitutively expressed in neonatal rodent cerebellum (Ni et al., 1997).

In cultured cerebellar granule cells (CGN), chronic depolarization by potassium promotes cell survival (Gallo et al., 1987; Balázs et al., 1988). This effect is observed only during critical periods of time, i.e., after 5–7 days in vitro (DIV), corresponding to the time when CGN receive presynaptic glutamatergic inputs from mossy fibers during development in vivo. Also, when CGN cultured in 25 mM $[K^+]e$ (K25) during 6–8 DIV are transferred to a medium containing 5 mM $[K^+]_e$ (K5) most cells die after 24–48 hr showing morphological features of apoptosis (Gallo et al., 1987; D'Mello et al., 1993, 1997; Yan et al., 1994; Nardi et al., 1997; Morán et al., 1999). It has been demonstrated that caspase-3 plays a critical role in CGN death induced by K5 (Morán et al., 1999). Death of CGN transferred to K5 can be prevented by increasing intracellular Ca^{2+} levels $[Ca^{2+}]_i$, by addition of a Ca^{2+} ionophore or by activation of Ca^{2+}-permeable ionotropic glutamate receptors. On the other hand, death of CGN chronically

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*Correspondence to: Julio Morán, Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México City 04510, D.F., Mexico. E-mail: jmoran@ifisiol.unam.mx

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cultured in K25 can be elicited by inhibiting their voltage-gated Ca^{2+} channels (Kingsbury and Balàzs, 1987; Balàzs et al., 1988; Pearson et al., 1992; Copani et al., 1995; Morán et al., 1999). These observations suggest that K25 enhances CGN survival by augmenting Ca^{2+} influx and therefore that $[Ca^{2+}]_i$ is probably the first signal responsible for the survival of CGN (Kingsbury and Balàzs, 1987; Balàzs et al., 1988; Morán et al., 1999).

Some studies have found that changes in $[Ca^{2+}]_i$ are related to oxidative stress and that reactive oxygen species (ROS) constitute another critical element in the process of apoptotic cell death. ROS have been proposed to act as inductors of necrotic and apoptotic cell death in a variety of models (Greenlund et al., 1995; Stephen et al., 1998; Stuart et al., 1998; Keller et al., 1998; Kruman et al., 1998). In vivo and in vitro studies have shown a marked increase in ROS levels during apoptotic cell death. Mitochondria seem to play a critical role in this process by contributing with the generation of ROS, particularly superoxide anion (Azbill et al., 1997; Atlante et al., 1998; Green et al., 1998). Cytosolic ROS could also participate in the mechanism involved in apoptosis, however, particularly through the NADPH-oxidase activation, it has been proposed in sympathetic neurons (Tammariello, 2000). In several preparations, including CGN, antioxidant treatment prevents cell death by using scavengers of hydroxyl radicals (like DMSO or mannitol) or with enzymatic conversion of superoxide radicals to hydrogen peroxide by copper/zinc or manganese superoxide dismutase (SOD) addition, among others (Schultz et al., 1996; Atlante et al., 1998; Park et al., 1998).

Intracellular Ca^{2+} levels play an important role in cell death. It has been proposed that under conditions of excessive neuronal stimulation, $[Ca^{2+}]_i$ homeostasis is altered resulting in a mitochondrial Ca^{2+} overload and a subsequent ROS production and death (Atlante et al., 1998; Keller et al., 1998; Stuart et al., 1998). Some immature neurons, including CGN, however, require relatively high levels of $[Ca^{2+}]_i$ to survive (Kingsbury and Balàzs, 1987; Balàzs et al., 1988; Johnson et al., 1992; Pearson et al., 1992; Copani et al., 1995) and a reduction of $[Ca^{2+}]_i$ in CGN induces activation of caspase-3 and apoptotic cell death (Morán et al., 1999). Thus, although the exact role of $[Ca^{2+}]_i$ and ROS is unknown it is clear that apoptosis is an event influenced by these two conditions. Thus, in this study we analyzed the functional and temporal relationship among oxidative stress, change of $[Ca^{2+}]_i$ and caspases activity during the apoptotic death of CGN induced by K5 to understand the mechanisms involved in this process.

MATERIALS AND METHODS

Materials

Fetal calf serum and penicillin/streptomycin were from GIBCO (Grand Island, NY). Poly-L-lysine (mol. wt. >300,000), trypsin, DNAse and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). Reagents for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad (Hercules, CA), and polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA). Protein molecular weight markers and annexin V were from Boehringer (Mannheim, Germany). Antibodies against caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase substrates and inhibitors were from Peptides International (Louisville, KY). Fura-2 acetoxymethyl ester (fura-2/AM), Hoechst 33258 and Pluronic F-127 were from Molecular Probes (Eugene, OR). Dihydrorhodamine-123 (DHR) and 2′7′Dicholorodihydrofluorescein diacetate (H_2DCFD), catalase, SOD, mannitol, DMSO, xanthine and xanthine oxidase were for Sigma.

Cerebellar Granule Neuron Cultures

CGN cultures were prepared as previously described (Morán and Patel, 1989). Briefly, cell suspensions dissociated from 8-day-old rat cerebellum were plated at a density of 265×10^3 cells/cm^2 in plastic dishes previously coated with poly-L-lysine (5 μg/ml) and over coverslips of 25 × 25 mm. The 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 μg/ml streptomycin. This medium is referred in text as K25. The cultures dishes were incubated at 37°C in a humidified 5%CO_2/95% air atmosphere. Cytosine arabinoside (10 μM) was added 20 hr after seeding. These CGN cultures contained approximately 95% neurons. Cells were maintained for 6–8 days in vitro (DIV). In some experiments, CGN were transferred to a serum-free medium without cytosine arabinoside containing 5 or 25 mM KCl with or without the different treatments: DMSO 0.1, 0.5, 1 and 1.5%, SOD (Cu/Zn and Mn) 1, 5, 10 and 30 mU/ml, catalase 0.1, 1 and 10 mU/ml, mannitol 10 mM, as described in Results.

Immunoblots

CGN were washed twice with 37°C phosphate-buffered saline (PBS), homogenized in lysis buffer [50 mM HEPES; 0.5 mM EDTA; 1% (v/v) NP-40; 0.25% (w/v) SDS; 0.5 mM EGTA; 150 mM NaCl; 10 mM DTT; 1mM PMSF, 1 mM NaF; 2 μg/ml aprotinin; 1 μg/ml pepstatin; and 5 μg/ml leupeptin], and centrifuged at 3,000 × *g* for 10 min at 4°C. Homogenates (50–100 μg protein per lane) were subjected to SDS-PAGE (Laemmli, 1970). The resolved proteins were transferred to PDVF membranes at 50 mV for overnight. The membranes were blocked for 3 hr with 5% nonfat dry milk in TPBS (PBS, 0.1% (v/v) Tween 20) and treated with a primary antibody against caspase-3 or caspase-7 followed by an alkaline phosphatase-linked secondary antibody. Bands were visualized using enhanced chemiluminescence according to the manufacturer's protocol. Polyclonal antibodies against caspase-3 reacts with caspase-3 and its p17 and p20 subunits and was raised against the full-length precursor form of caspase-3 of human origin. Polyclonal antibody against caspase-7 reacts with the proenzyme and its p25 and p19 subunits and was raised against the full-length precursor form of caspase-7 of human origin.

$[Ca^{2+}]_i$ Measurements

CGN $[Ca^{2+}]_i$ was quantified using the fluorescent calcium indicator fura-2/AM, as previously described (Itoh et al.,

1998). Briefly, cells grown on coverslips for 6–8 DIV were incubated with 5 μM fura-2/AM and 0.02% Pluronic F-127 for 1 hr in a K25 medium and washed two times. The coverslips were placed on a perfusion chamber (RC-25, Warner Instrument Corp, CT) and perfused with a K25 or a K5 plus antioxidants medium during measurement. Cells were alternatively illuminated with a Xe arc lamp through 340 nm and 380 nm excitation filters. Emission fluorescence images (510 nm), obtained with an epifluorescence inverted microscope (Nikon Diaphot TMD, Nikon Corporation, Japan) were converted to digital data by an image-processing system (BioLase Imaging System, Newton, MA). The key elements of the fluorescence Ca^{2+} imaging system were a high numerical aperture UV objectives (Nikon UV-F 40× oil immersion and 100× glycerol immersion, 1.4 and 1.3 NA respectively), an intensified charge coupled device camera (c2400-87, Hamamatsu Bridgewater, NJ) and the BioLase Imaging System running under their FL-2 software. The system allows real-time simultaneous acquisition of fluorescence measurements from multiple areas of interest placed on individual cells. $[Ca^{2+}]_i$ in individual neurons was calculated from the ratio (R) of 340 nm to 380 nm excitation wavelength, using the following formula:

$$[Ca^{2+}]_i = bK_d(R - R_{min})/(R_{max} - R)$$

where K_d is the dissociation constant of fura-2/AM; b is the ratio of fluorescent signals at 380 nm for Ca^{2+}-free and Ca^{2+}-saturated dye; R_{min} is R in the absence of external Ca^{2+}; R_{max} is R in saturating $[Ca^{2+}]_i$. These parameters were determined by in vitro calibration using EGTA and ionomycin.

Cell Viability

Cell viability was estimated by MTT assay at 12–48 hr after transferring cells to K5 medium. MTT (0.1 mg/ml) was added to the CGN and incubated for 15 min at 37°C. After removal of medium containing the remaining MTT, 100% DMSO was added to the dishes. After incubation for 15 min at room temperature in darkness, formazan blue formed from MTT was quantified spectroscopically at 560 nm excitation wavelength. As previously shown (Balàzs et al., 1990; Morán et al., 1999), there was a very good correspondence between the capacity of cultures to form formazan blue and the protein and DNA content and the proportion of neurons that appeared intact by phase-contrast microscopy.

Cell viability was also assessed by evaluating the capability of CGN to diesterify and to retain fluorescein diacetate (FDA) in their cytoplasm. Cells were washed twice, incubated with 5 μg/ml of FDA in culture medium for 20 min at 37°C and then cells were washed and mounted in perfusion chamber for analysis by epifluorescence microscopy using an excitation and emission wavelength of 485 and 520 nm, respectively. Propidium iodide (PI) was used to identify dead neurons. Cells were incubated with 10 μM PI for 20 min at 37°C and then cells were analyzed with an epifluorescence microscope using a fluorescence filter with an excitation of 450 nm and emission of 510 nm.

Nuclear Condensation Detection by Hoechst Dye

Cells plated on coverslips at a density of 265×10^3 cells/cm^2 were cultured in a K25 medium for 6–8 DIV and then switched to a K5 medium plus antioxidants. After 24 hr, cells were fixed in 4% fresh paraformaldehyde at 4°C for 30 min, then incubated with 0.01% Hoechst 33258 in PBS for 10 min and mounted with methanol-glycerol 1:1 v/v. Cultures were examined with an UV objective (100× glycerol immersion, 1.3 NA) in an epifluorescence microscope (Nikon, UV BA-2 filter and a barrier filter of 520 nm using a 340 nm excitation and 460 nm emission wavelengths).

Caspase Activity

After 4–24 hr transferring to K5, CGN were washed with PBS and homogenized in lysis buffer, diluted 1:1 (v/v) with glycerol and stored at ±70°C. Caspase activities were assayed by a fluorometric method (Thornberry, 1994) with a luminescence spectrometer (Spectronic Instruments, SLM Aminco-Bowman, Rochester, NY), using different substrates for each caspase. Ac-DEVD-AMC was used as substrate to detect caspase-3 activity, Ac-YVAD-AMC for caspase-1, Ac- IETD-AMC for caspase-8 and Ac-LEHD-AMC for caspase-9. Activities were followed for 15–20 min after addition of substrate (25 μM) and cell homogenate (60–90 μg/ml) in a standard solution [100 mM HEPES; 10% (w/v) sucrose; 0.1% (w/v) CHAPS; 10 mM DTT; 1 mM EDTA; 1 mM PMSF; 1 mM NaF; 2 μg/ml aprotinin; 1 μg/ml pepstatin; and 5 μg/ml leupeptin]. Results are expressed as YVAD, DEVD, IETD and LEHD cleaved as change in fluorescence intensity/hr/mg protein.

Measurements of Mitochondrial and Cytosolic ROS Formation

Cells plated on coverslips were cultured in a K25 medium for 6–8 DIV and then incubated for 30 min with 10 μg/ml DHR-123; this non-fluorescent dye is sequestered by mitochondrion, oxidized and converted into a fluorescent compound. For cytosolic ROS formation, cells were incubated for 30 min with 10 μg/ml of H_2DCFD; this non-fluorescent dye is deacetylated in the cytoplasm by stearates and converted into a fluorescent compound by oxidation. After incubation, cells were washed with Locke's medium (1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 5.5 mM glucose, 10 mM HEPES, 3 mM $NaHCO_3$, 120 mM NaCl and 25 mM KCl), and the coverslips were mounted in a perfusion chamber (Warner Instruments, Mod. RC-25, Hamden, CT). Fluorescence was detected spectrofluorometrically (SLM-AMNICOR, Rochester, NY) at a 488 nm excitation and 530 nm emission wavelengths for DHR-123; and at a 480 nm excitation and 530 nm excitation wavelengths for H_2DCFD. In all cases, cells were exposed only for 30 sec to UV light using different samples at the indicated times after K5 treatment. Each sample was compared with control culture (K25) exposed in parallel to UV light. To discount fluorescence generated by UV photo-oxidation, the delta of fluorescence obtained in the 30 sec period in K25 cells was subtracted from the fluorescence obtained in the experimental sample measured at the same time. Therefore, different samples from the same culture were used to cover the whole period of time indicated in the figures. The delta of fluorescence in K25 (i.e., fluorescence generated by UV photo-oxidation)

was always negligible as compared with the fluorescence generated in the K5 samples. In other experiments, cells were incubated with dye after 1–8 hr of medium change from K25 to K5 medium and then fluorescence was measured as detailed above. Individual neurons were analyzed using an image-processing system (BioLase Imaging System, Newton, MA). The key elements of the fluorescence imaging system were the same as described for Ca^{2+} measurement.

Translocation of Phosphatidylserine (PS)

Cells were plated on coverslips at density to 265×10^3 cells/cm^2 and incubated for 6–8 DIV. At this time, cultures were incubated in a K5 serum-free medium plus antioxidants. After medium change, cells were washed with PBS at the indicated times (1–8 hr), and then incubated for 15 min with Annexin V-FITC. Cells were washed with PBS and mounted with methanol-glycerol 1:1 v/v. Preparations were observed under epifluorescence microscopy using a rhodamine filter (Martin et al., 1995). The fluorescence imaging system was the same as described for Ca^{2+} measurement. Results are expressed as arbitrary units of fluorescence intensity (20 neurons by field).

Statistical Analysis

Data were expressed as means ± SDS, and statistical significance of the results was determined by one-way analysis of variance (ANOVA) followed by Fisher's test post-hoc, with statistical significance set at $P > 0.05$.

RESULTS

Antioxidants Promote Cell Survival of CGN in K5 Medium

In agreement with previous reports (D'Mello et al., 1997; Ishitani et al., 1997; Nardi et al., 1997; Villalba et al., 1997), when CGN cultured in K25 for 8 days were transferred to K5, more than 45 and 80% CGN died after 24 and 48 hr, respectively, as measured by MTT transformation (Fig. 1A). These results were similar to those obtained using FDA to label living cells (82.8 ± 8.9% of FDA positive cells after 12 hr; 48.6 ± 5.2% after 24 hr; and 32.8 ± 3.1 after 48 hr). Cell viability measured 24 hr after transferring cells to K5, was unaffected if CGN were returned to a K25 medium within the first 3 hr (Fig. 1B). After 4 hr of K25 replacement, less than half of the population could be rescued and after 6–8 hr of K25 replacement all cells died (Fig. 1B). Thus, under these conditions, CGN are committed to die after 4 hr of K5 treatment. Also, these observations were supported by the morphological appearance of CGN when analyzed by phase contrast microscopy (Fig. 2A,B). In line with previous studies, the observed CGN death induced by K5 showed characteristics of apoptosis as evidenced by the nuclear condensation in K5 cells stained with Hoechst after 24 hr (Fig. 2E,F) and the translocation of phosphatidylserine in K5 cells labeled with annexin V (Fig. 3A). The translocation of phosphatidylserine (PS) showed an increase after 3 hr of transferring CGN from K25 to K5 media, reaching a maximal at 5 hr, when it remained without change for the following 6 hr (Fig. 3A).

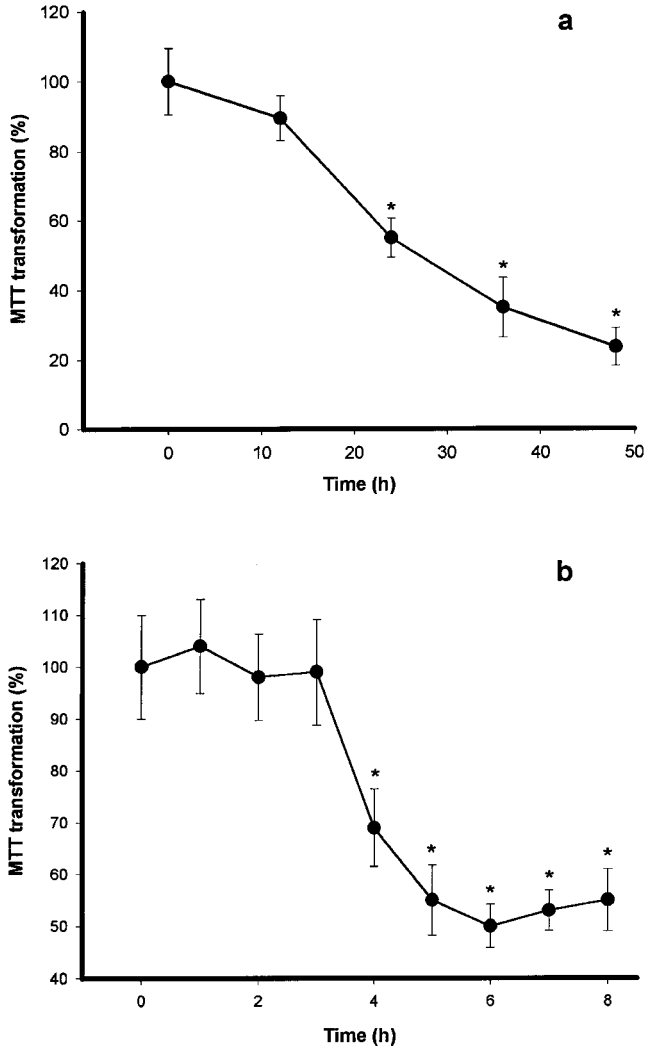

Fig. 1. Time course of CGN death induced by low $[K^+]_e$. Cells were switched from 25 mM KCl to a 5 mM KCl medium and cell survival was estimated as MTT transformation. **A:** Time course of CGN survival after change to K5 medium. **B:** CGN survival measured 24 hr after switching to a K5 medium and then returned to K25 medium at the indicated times. Note that after 4 hr in a K5 medium CGN cannot be rescued by K25. Data are mean ± SEM of 9 independent experiments. *Significantly different from control (time = 0) ($P < 0.05$).

Several antioxidants that act through different mechanisms, were tested on CGN death induced by K5 after 24 hr. In agreement with previous studies (Atabay et al., 1996; Schulz et al., 1996), it was found that the antioxidant enzymes Cu/Zn SOD (1–30 mU/ml) and catalase (0.1–10 mU/ml) were able to protect from cell death by about 75% and 56%, respectively, when added simultaneously to the change of medium (Fig. 2C,D). Chromatin condensation was also markedly decreased in cells switched to K5 plus the antioxidant enzymes as evidenced by nuclear staining with Hoechst (Fig. 2G,H). The mitochondrial form of SOD, MnSOD used at 10 mU/ml was also very efficient by inhibiting CGN death around 60% (not shown). Other antioxidants acting as scavengers of

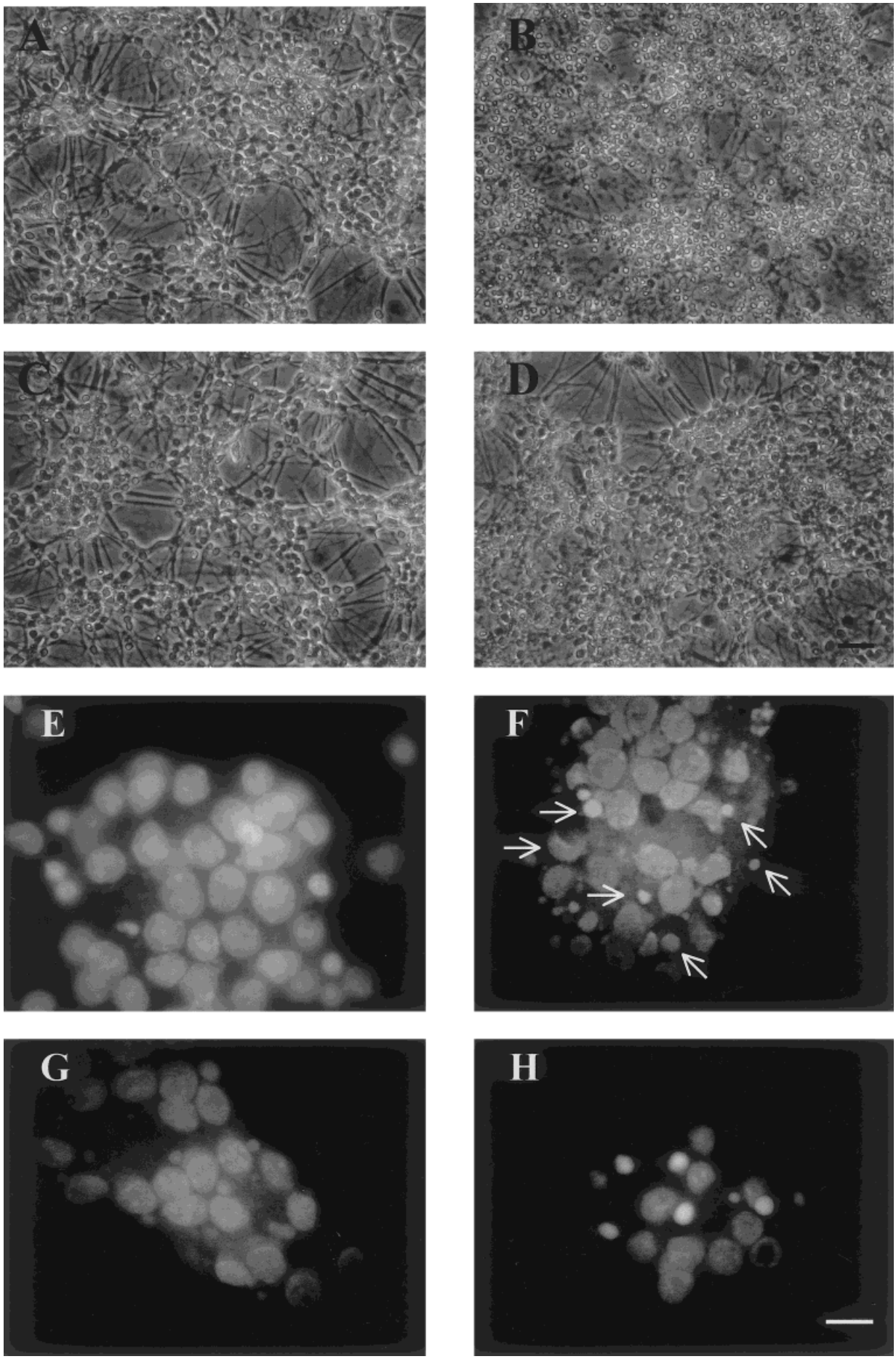

Figure 2.

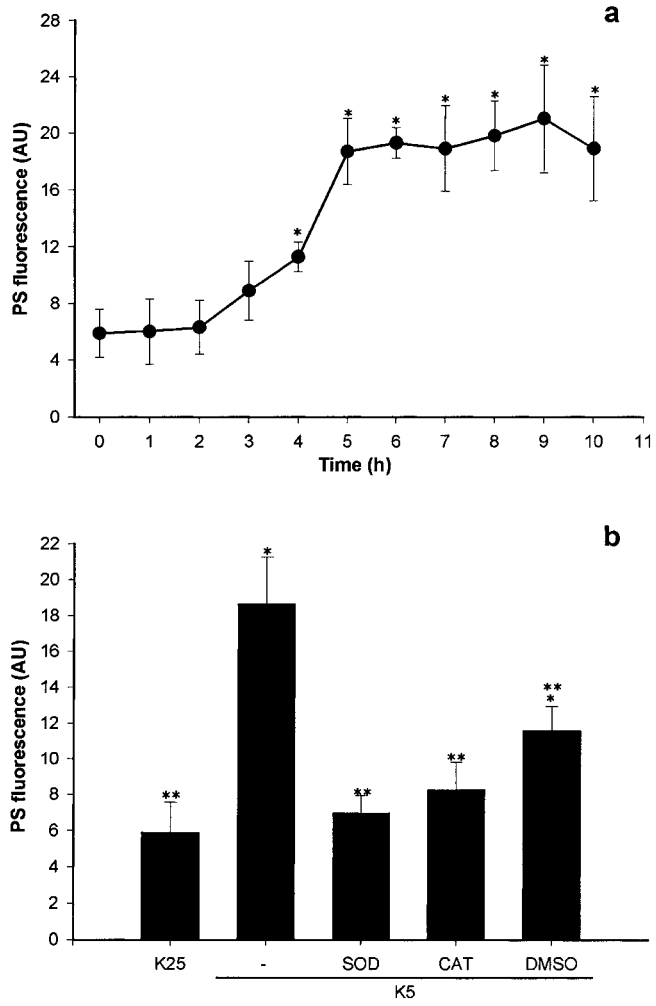

Fig. 3. Effect of antioxidants on phosphatidylserine (PS) translocation induced by low $[K^+]_e$ in CGN. CGN were grown by 7 DIV in a K25 medium, and cells were switched to a K5 medium with or without antioxidants. PS translocation was evaluated as annexin-V binding as described in Methods and Materials. **A:** Time course of PS translocation in CGN transferred from a K25 medium to a K5 medium; **B:** PS translocation in CGN maintained 8 hr in a K5 medium plus 10 mU/ml Cu/ZnSOD (SOD), 1 mU/ml catalase (CAT) or 1.0% DMSO. Values are means ± SEM of determinations from a total of at least 30 cells from five independent experiments. Significantly different from *control (K25 or time = 0) and **K5 ($P < 0.05$).

hydroxyl radicals, peroxynitrites and peroxides like DMSO (1%) or mannitol (10 mM) (not shown) also significantly inhibited cell death by 40–50%. Glutathione (5–10 mM) protected from cell death only marginally and other antioxidants like α-tocopherol (50 mU/ml) or *N*-acetyl cysteine (0.1–1.0 mM) did not exert any effect on CGN survival (not shown).

All antioxidants tested, including Cu/Zn and Mn-SOD, catalase and DMSO added at the time of media change, prevented the phosphatidylserine translocation (Fig. 3B). These results suggested that ROS production is an event occurring earlier than PS translocation during cell death of CGN.

Antioxidants Suppress ROS Formation Induced by K5 at Different Times

When cells are changed from a K25 medium to a K5 medium both cytosolic and mitochondrial ROS are formed at three different times after switching (Fig. 4). Around 5–6 min after transferring CGN to a K5 medium a rise in cytosolic ROS is detected as DCF-fluorescence (Fig. 4A). Similarly, a significant increase in mitochondrial ROS, detected as DHR-123 fluorescence, is observed after 40 min of transferring cells to K5 medium (Fig. 4B). A late increase of both cytosolic and mitochondrial ROS is also observed after 3 hr, which reached a maximum after 4 hr of medium change (Fig. 4C,D). When CGN switched to a K5 medium were simultaneously treated with Cu/ZnSOD (10 mU/ml), catalase (1 mU/ml) or DMSO (1%) both the early and late peaks of cytosolic and mitochondrial ROS were completely suppressed (Fig. 4).

To elucidate which of the observed ROS produced are involved in the process of PND of CGN, we switched cells to a K5 medium followed by addition of antioxidants like Cu/ZnSOD, catalase or DMSO at different times (0, 2 and 5 hr) of switching (Fig. 5). Under these conditions we found that all antioxidants were equally efficient in protecting CGN from death when added simultaneously (0 hr), after 30 min (not shown) or 2 hr after medium change. In contrast, when antioxidants were added to cultures after 5 hr of switching to K5 none of these agents were able to rescue cells from death (Fig. 5). Thus suggesting that only the late ROS peak (after 3–4 hr) may play a direct role in the process of cell death.

Intracellular Calcium Levels Are Not Modified by K5 and Antioxidants Treatment

As we previously showed (Morán et al., 1999), when CGN maintained in a K25 medium are transferred to a K5 medium, $[Ca^{2+}]_i$ is immediately reduced from about 280 nM to 50 nM (Morán et al., 1999) (Fig. 6A). This reduction of $[Ca^{2+}]_i$ is kept constant (around 50 nM) in all the neurons evaluated during the first 4 hr after the K5 treatment (Fig. 6D, insert). After 24–48 hr a fraction of the cells in K5 still showed the observed low levels of

Fig. 2. Effect of antioxidants on the morphology of CGN switched to a K5 medium. CGN were grown by 7 DIV in a K25 medium, switched to a K5 medium and after 24 hr were observed by phase contrast (**A–D**) or fixed, incubated with Hoechst dye and observed in a fluorescence microscope (**E–H**). A,E: CGN switched to a K25 medium (control). B,F: CGN switched to a K5 medium after 24 hr. C,G: CGN switched to a K5 medium plus Cu/ZnSOD (10 mU/ml) for 24 hr. D,H: CGN switched to a K5 medium plus catalase (1 mU/ml) for 24 hr. (A–D) Scale bar = 50 μm (A–D); scale bar = 20 μm (E–H).

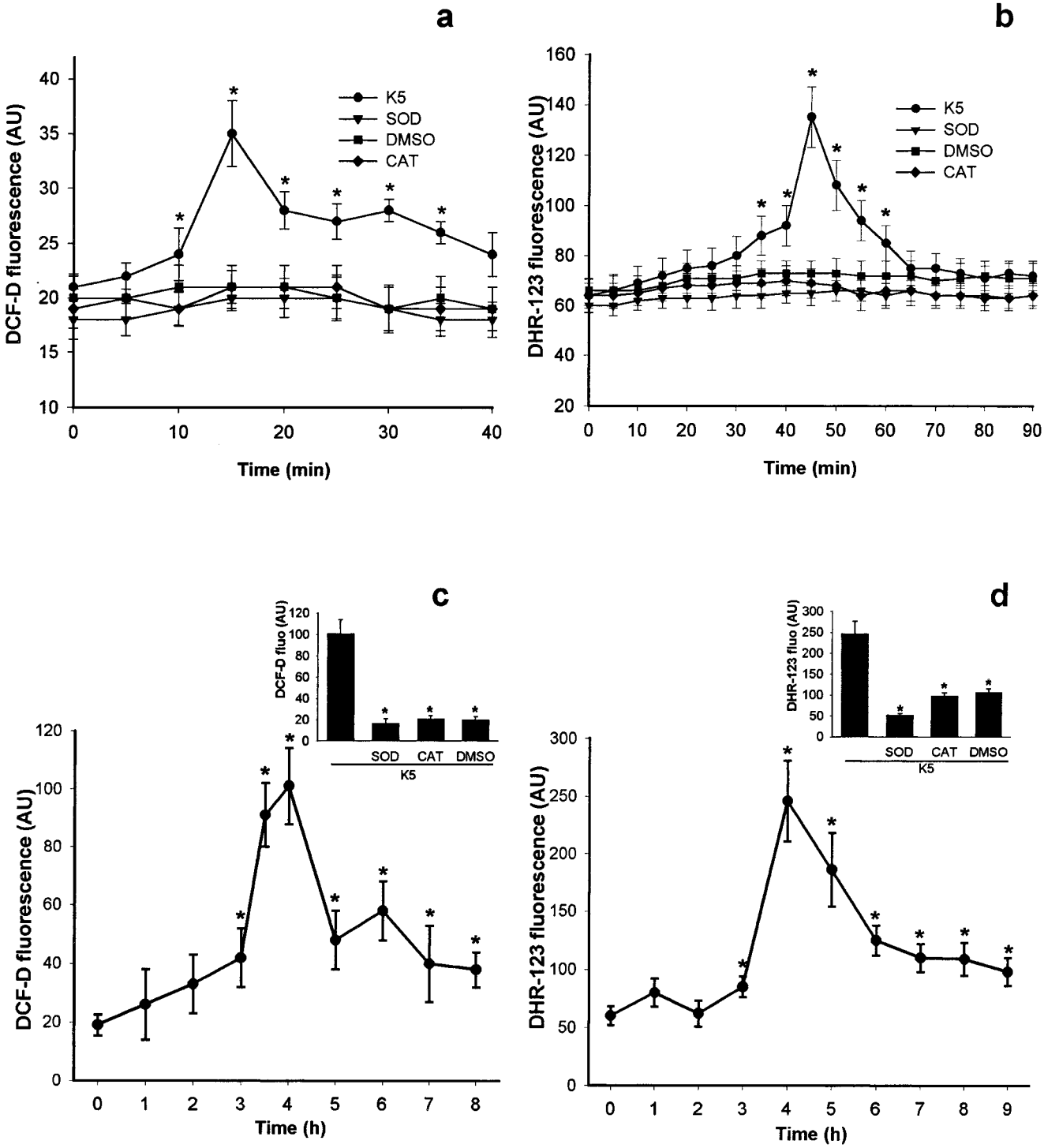

Fig. 4. Time course of ROS formation induced by low $[K^+]_e$ in CGN. ROS formation was measured as fluorescence intensity of DCF to detect cytosolic ROS (**A,C**) or DHR-123 to detect mitochondrial ROS (**B,D**) as detailed in Methods and Materials. Cells were incubated with the probes and fluorescence was detected spectrofluorometrically. Fluorescence measurement was followed for several periods of times. Individual neurons were analyzed using an image processing. Values are means ± SEM of 4–6 independent experiments. *Significantly different from control (time = 0) ($P < 0.05$).

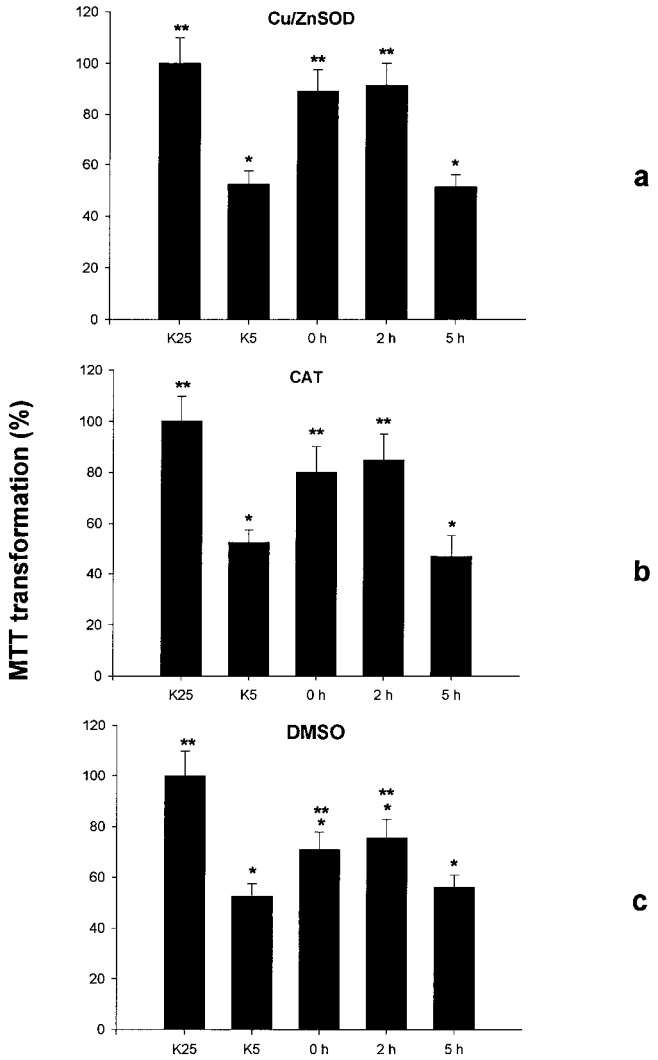

Fig. 5. Effect of antioxidants on CGN death induced by low $[K^+]_e$. CGN were grown for 7 DIV in a K25 medium, switched to a K5 medium added with 10 mU/ml Cu/ZnSOD **(A)** 1 mU/ml catalase; **(B)** or 1.0% dimethyl sulfoxide; **(C)** after 0, 2 hr or 5 hr of medium change and viability was measured as MTT transformation after 24 hr. Bars are means ± SEM of nine independent experiments. Significantly different from *K25 and **K5 ($P < 0.05$).

$[Ca^{2+}]_i$. In contrast, a second population of cells exhibited variable increased $[Ca^{2+}]_i$ levels probably corresponding to cells that are close to dying (not shown). The presence of 1 mU/ml catalase, 10 mU/ml Cu/ZnSOD or 1.0% DMSO in the K5 medium did not modify the mentioned reduction in $[Ca^{2+}]_i$ (Fig. 6B–D). Four hours after the change to K5 medium $[Ca^{2+}]_i$ none of the tested antioxidants exerted a significant change on the observed $[Ca^{2+}]_i$ (Fig. 6D, insert). Thus, $[Ca^{2+}]_i$ do not seem to be directly related to the late ROS peaks observed around 4 hr after switching and the effect of antioxidants seems not to be mediated through a modification in $[Ca^{2+}]_i$.

Antioxidants Inhibit the Activity of Caspases Activated by K5

Caspase activity was measured using substrates like DEVD-AMC for caspase-3; IETD-AMC for caspase-8 and LEDH-AMC for caspase-9. We demonstrated for the first time that CGN transferred to K5 show a significant increase of caspase-8 activity, which peaked after 6–8 hr (Fig. 7A). Cell death induced by K5 was partially prevented (about 40%, after 36 hr) in the presence of the caspase-8 inhibitor IETD-CHO at 300 μM. On the other hand, as others (Shultz et al., 1996; Atlante et al., 1998) and we (Morán et al., 1999) have shown, DEVD-AMC cleavage was markedly increased in cells changed to a K5 medium (Fig. 8A). DEVD cleavage reached a maximum after 8–9 hr showing a marked decrease after 12 hr (Fig. 8A). This activation was markedly higher (about 10 times) than that observed for caspase-8 (about 2 times). As DEVD can be cleaved by caspase-3 and caspase-7, we confirmed by Western blot analysis the processing of caspase-3 in cells switched to K5 (Fig. 8C). This result, however, does not rule out the possibility that caspase-7 could be also involved in the observed DEVD cleavage. Two other caspases were also measured in cells transferred to K5 medium: caspase-1 (measured as YAVD cleavage) (not shown) and caspase-9 (measured as LEHD cleavage) (Fig. 7A). None of these caspases showed any activation by K5 at the times measured (2–24 hr).

Caspase-8 activity, induced by K5 was completely abolished by SOD, DMSO and catalase (Fig. 7B). SOD treatment even reduced the basal activity observed in K25 cells (Fig. 7B). Similarly, DEVD cleavage induced by K5 was also markedly inhibited by the action of all antioxidants tested, although in contrast to caspase-8, inhibition was not complete (Fig. 8B). SOD at 10 mU/ml reduced by 82% the DEVD cleavage, whereas catalase and DMSO also partially inhibited DEVD cleavage by 73% (Fig. 8B). In all cases, the addition of the antioxidants at 30, 60, 90 (not shown) and 120 min (Fig. 8B) after switching did not modify the effect of the tested antioxidants. Although the antioxidants were very efficient in inhibiting the activity of caspase-3, the processing of the proenzymes of both caspases was inhibited only marginally. Figure 8C shows that the processing of the caspase-3 proenzyme was partially inhibited in K5 cells treated with SOD and catalase, but not DMSO.

Oxidative Stress Induces CGN Death and Caspase-3 Activation

To study the effect of oxidative stress in CGN maintained in K25 (i.e., high levels of $[Ca^{2+}]_i$) we measured CGN survival and DEVD-cleavage in cells treated with H_2O_2 or xanthine/xanthine oxidase (XA/XO), two conditions known to induce ROS. Figure 9A shows that both conditions lead to CGN death, although H_2O_2 treatment induces a 50% cell death after 1.5 hr and XA/XO after 5 hr. Interestingly, in contrast to H_2O_2, treatment with XA/XO increased caspase-3 activity after 2 hr (Fig. 9B).

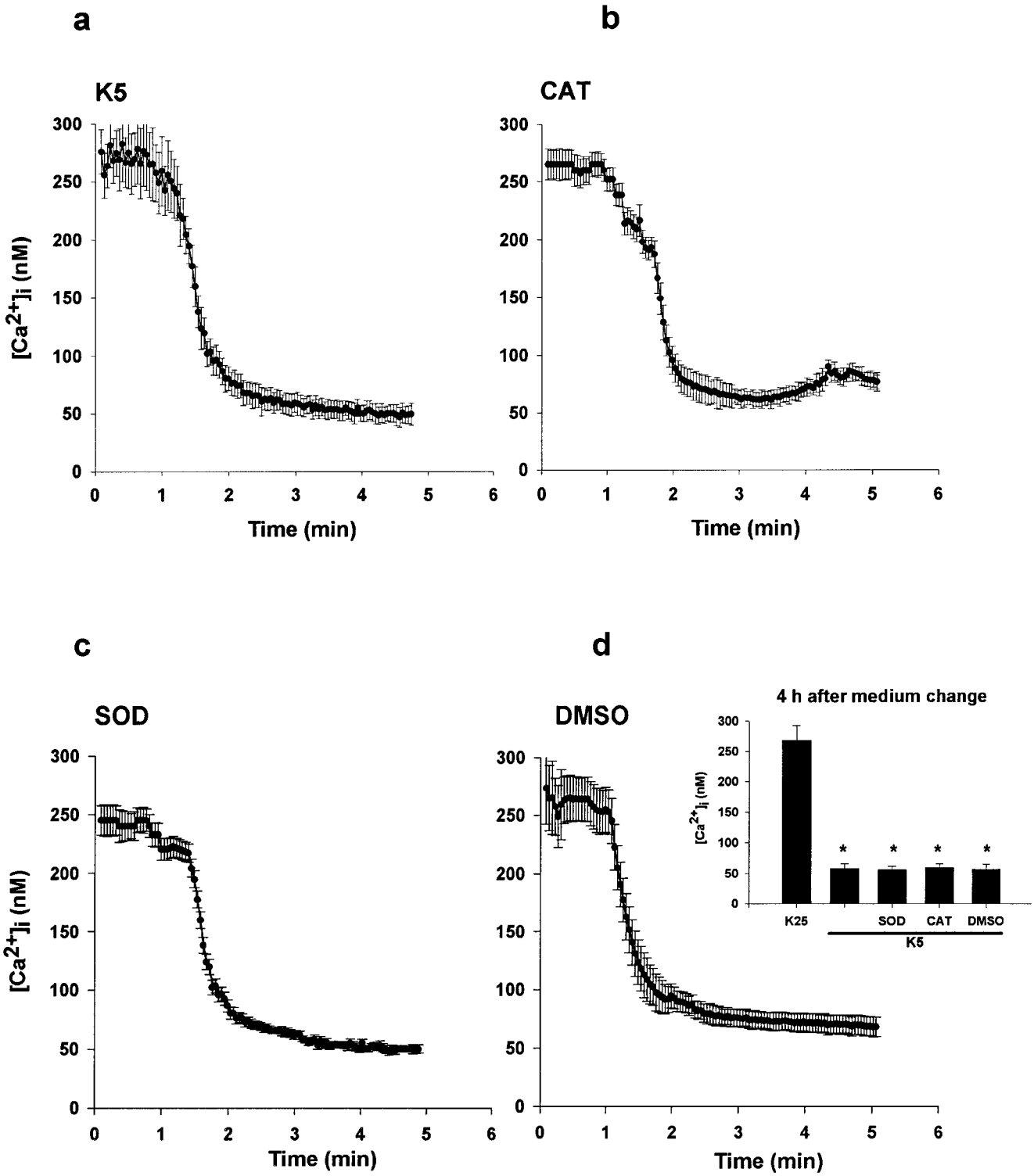

Fig. 6. Effect of antioxidants on $[Ca^{2+}]_i$ in CGN switched to low $[K^+]_e$. CGN were preloaded with fura-2/AM in a K25 medium, perfused with a K25 medium for 20 min and then transferred to a K5 medium (min 1) in the presence of antioxidants. **A:** Control; **B:** 1 mU/ml catalase; **C:** 10 mU/ml Cu/ZnSOD (SOD); **D:** 1.0% DMSO. **Inset:** $[Ca^{2+}]_i$ in CGN maintained in low $[K^+]_e$ for 4 hr in the presence of antioxidants at the same concentrations as above. *Significantly different from K25 ($P < 0.05$). In all cases, values are means ± SEM of determinations from at least 20 neurons from four independent experiments.

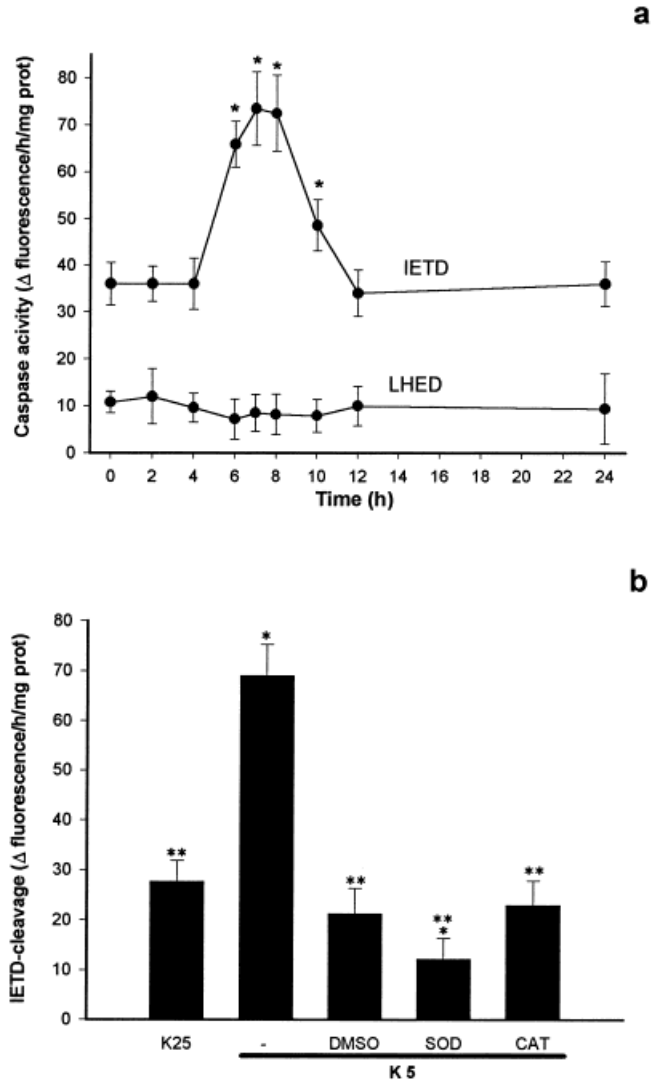

Fig. 7. Effect of antioxidants on caspases activity induced by low $[K^+]_e$ in CGN. Cells were grown by 7 DIV in a K25 medium and then switched to a K5 medium during 0–24 hr (**A**), or 8 hr (**B**), and cell homogenates were processed for IETD-AMC or LEHD-AMC cleavage by a fluorometric procedure, as detailed in Methods. A: Time course of ac-IETD-AMC or LEHD-AMC cleavage. Values are means ± SEM of 4 independent experiments. B: Effect of antioxidants on IETD-AMC cleavage induced by low $[K^+]_e$ after 7 hr. 1.0% DMSO, 10 mU/ml Cu/ZnSOD (SOD) or 1 mU/ml catalase (CAT) were added to CGN simultaneously to K5. Scale bars = means ± SEM of six independent experiments. Significantly different from *K25 and **K5 ($P < 0.05$).

The participation of caspases in the CGN death induced by XA/XO was further supported by the observed inhibitory effect of a general blocker of caspases activity on CGN death (Table I). When cultures were treated with 150 μM z-VAD, CGN death induced by XA/XO was significantly decreased by 50%. In contrast, cell death induced by H_2O_2 was unaffected by treatment with the caspase inhibitor (Table I).

DISCUSSION

Caspases have been shown to play a key role in apoptotic cell death (Chen et al., 1998; Ni et al. 1998) and among them caspase-3 is the most prominently involved in apoptosis in neural tissues (Martin and Green, 1995; Cohen et al., 1997). On the other hand, $[Ca^{2+}]_i$ and ROS have been suggested to play a central role in the initiation and execution of neuronal apoptosis (Johnson et al., 1992; Pearson et al., 1992; Copani et al., 1995; Atlante et al., 1998; Keller et al., 1998; Krohn et al., 1998; Morán, et al., 1999). In this study we analyzed the role of oxidative stress and its relationship to $[Ca^{2+}]_i$ and caspase activation in neuronal apoptotic cell death using CGN apoptotic death as a model of study. When CGN grown in a medium containing 25 mM KCl are transferred to a 5 mM KCl medium, cells die with characteristics of apoptosis, including nuclear condensation, DNA fragmentation, etc. (D'Mello et al., 1993, Yan et al., 1994; Schulz et al., 1996; Nardi et al., 1997; Morán et al., 1999). In this study we also confirm that K5 activate caspase-3 and we demonstrated that this condition also induces caspase-8 activation.

Here, we confirmed previous observations that ROS, like superoxide anion, play a key role in apoptosis of CGN. We found that a variety of antioxidants are able to inhibit the CGN death process. Among the antioxidants tested, SOD was the most efficient followed by catalase and DMSO. Glutathione had only a marginal effect. SOD is known to metabolize superoxide anion, catalase removes peroxides and mannitol, DMSO and glutathione act as scavengers of hydroxyl anion, peroxynitrites and peroxides. Glutathione, in addition, participates in the reaction of removal of peroxides by glutathione peroxidase. Some preliminary studies in our laboratory have demonstrated that CGN loaded with dihydroethidium, a probe used to preferentially detect superoxide anion (Ishikawa et al., 1999), show a marked fluorescence after 4 hr of K5 treatment. All these results support the notion that superoxide anion is one of the main species involved in the process of CGN death and it is in agreement with other studies (Greenlund et al., 1995; Patel, 1996; Bisaglia et al., 2000).

On the other hand, we found that ROS levels markedly increased after changing CGN from K25 to K5 at different times in different cellular compartments. We used two fluorophores to detect cytosolic and mitochondrial ROS, H_2DCFD and DHR-123, respectively. An early increase in cytoplasmic ROS occurred at around 5–15 min after switching to K5. The source of these ROS was entirely cytoplasmic as no signal was observed with DHR-123. A second peak of ROS was detected in mitochondria after 30–40 min. This rise in ROS levels is coincident in time with an observed activation of SOD induced by the same experimental conditions (preliminary results), which further supports the primary formation of superoxide anion. Finally, in agreement with previous studies in this and other preparations (Greenlund et al., 1995; Schulz et al., 1996; Mattson, 1998), a peak of ROS

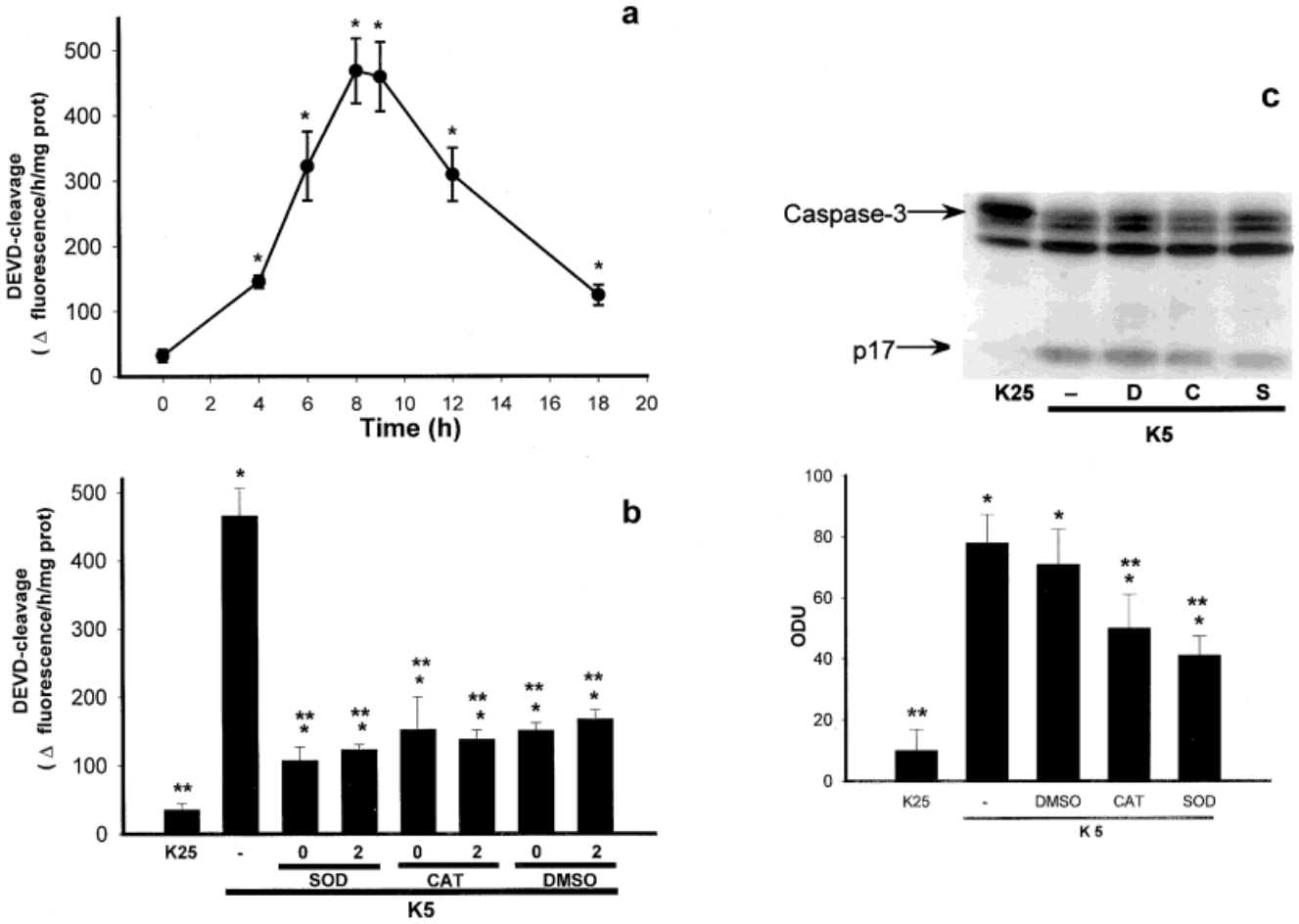

Fig. 8. Effect of antioxidants on caspase-3 activity induced by low $[K^+]_e$ in CGN. Cells were grown during 7 DIV in a K25 medium and then switched to a K5 medium for 0–20 hr (**A**), or 8 hr (**B**), and cell homogenates were processed for immunoblot or DEVD-AMC cleavage by a fluorometric procedure as detailed in Methods and Materials. A: Time course of DEVD-AMC cleavage. Values are means ± of 6 independent experiments. B: DEVD-AMC cleavage after 8 hr of medium change to K5 plus antioxidants. Antioxidants were added simultaneously (0) or 2 hr (2) after medium change. (**C**) Immunoblot of caspase-3 of cells treated with 1.0% DMSO (D), 1 mU/ml catalase (C) or 10 mU/ml Cu/ZnSOD (S) simultaneously to the switch to K5. p17, cleaved product of 17 kDa. Scale bar = means ± SEM of 3 determinations. Significantly different from *K25 and **K5 ($P < 0.05$).

levels was detected after 3–4 hr in both the cytoplasm and mitochondria compartments. Although we cannot rule out a simultaneous generation of ROS in the two compartments, it is possible that one peak could be involved in the generation of the second one. In this regard, a recent study demonstrated that neuronal death induced by NGF deprivation is dependent on cytosolic ROS production via NADPH-oxidase activation (Tammariello, 2000). In all cases, ROS production tends to return to normal levels after the peak production. This could be due to a cessation of the initial signal inducing the ROS production simultaneously to an activation of the endogenous antioxidant systems, like SOD. These two events would not be enough to prevent cell death.

The four peaks of ROS produced in CGN by K5 are completely inhibited by all antioxidant tested. Interestingly, the abolition by antioxidants of the first two ROS peaks (15 and 40 min) did not affect CGN death, whereas the inhibition of the late cytosolic and mitochondrial ROS peaks (3–4 hr) markedly reduced cell death. Thus, although we cannot rule out a possible influence of the early ROS peaks on the generation of the late ROS peaks, we can state that only the late ROS peaks (3–4 hr) seem to be critical for the CGN death process. In other models of neuronal death it has also been proposed that ROS is an early event in the process of apoptotic cell death (Guo et al., 1999; Ishikawa et al., 1999; Tammariello et al., 2000). In our system, the role of the ROS in the process of CGN apoptosis is not known. In this regard, our results show that ROS production is upstream to most of the early events during apoptosis and raises the possibility that they could serve as a signal to the initial phase of the apoptotic CGN death. This idea is supported by the fact that the time of appearance of the late ROS peaks corresponds to

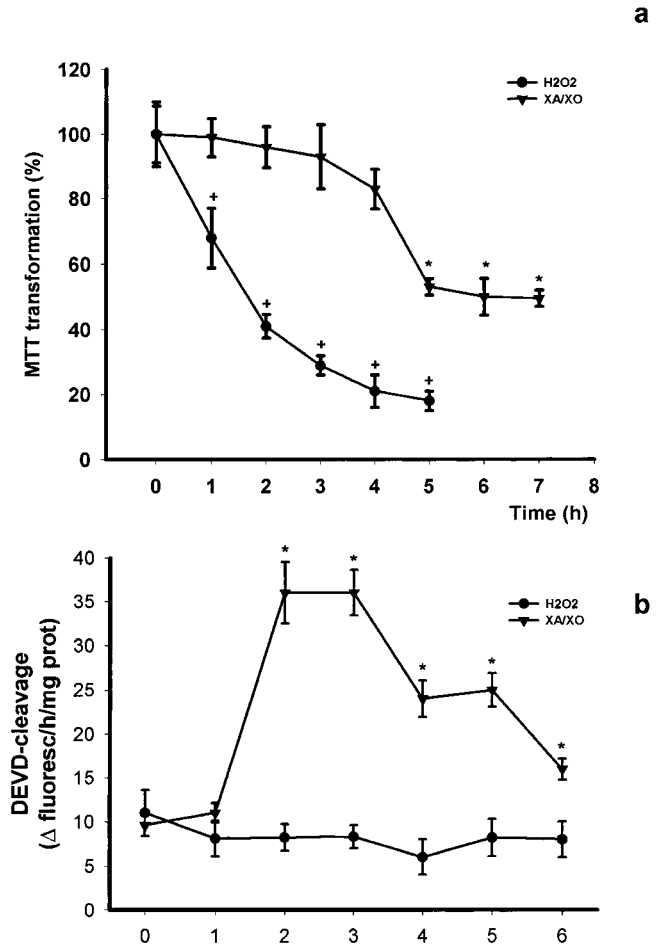

Fig. 9. Effect of H_2O_2 and XA/XO on cell viability and DEVD-cleavage in CGN. Cells were cultured for 7 DIV in a K25 medium and then treated with H_2O_2 (0.01%) or XA/XO (100 μM/ 45 mU). After the indicated times, MTT transformation (**A**) or DEVD-cleavage (**B**) was measured as indicated in Methods. Data are mean ± SEM of three independent experiments. ⋆,+Significantly different from control values ($P < 0.05$).

TABLE I. Effect of z-VAD on CGN Death Induced by XA/XO and H_2O_2†

Condition	MTT Transformation (%)
K25	100.0 ± 11.7
H_2O_2	20.0 ± 3.0⋆
H_2O_2 + z-VAD	21.2 ± 4.2⋆
XA/XO	52.5 ± 1.5⋆
XA/XO + z-VAD	73.2 ± 4.1⋆,⋆⋆

†Granule cells were cultured in a K25 medium for 7 days in vitro and then treated with H_2O_2 (0.01%) or XA/XO (100 μM/45 mU) with or without z-VAD (150 μM). After 5 hr MTT transformation was estimated as detailed in Methods. Results are means ±SD of 3 experiments.
⋆$P < 0.05$ compared to K25.
⋆⋆$P < 0.05$ compared to XA/XO.

the time when cells are committed to die, immediately before the translocation of phosphatidylserine and the activation of two caspases. Also, the cytosolic and mitochondrial peaks are only transient and last around 1 hr. Moreover, the presence of antioxidants blocks phosphatidylserine translocation and caspase activity. In this regard, we were able to induce in a short period of time (1–5 hr) a significant activation of caspase-3, nuclear condensation and apoptotic CGN death by increasing ROS in a xanthine/xanthine-oxidase system, but not H_2O_2, in cells maintained in a K25 medium. Thus suggesting that ROS, particularly superoxide anion, per se could directly induce some of the early apoptotic events in a Ca^{2+}-independent manner. The mechanism by which ROS could serve as a signal is not known, but they could act directly on a large number of groups of molecules. More recently, it has been proposed that ROS could also modulate the gene expression by acting on transcription factors in a variety of families like NF-κB, AP-1 and AP-2 (for review see Dalton et al., 1999).

It is well known that calcium plays a critical role in the process of apoptotic cell death of CGN (Kingsbury and Balàzs, 1987; Pearson et al., 1992; Castilho et al., 1999). In a previous study (Morán et al., 1999) we found that immature CGN require certain Ca^{2+} levels (about 300 nM) to support cell survival. At this Ca^{2+} level, both the activity and expression of caspase-3 are inhibited. When $[Ca^{2+}]_i$ is below a certain concentration (150 nM), cell death occurs concomitantly to a high activity of caspase-3. One question was to know the relationship between ROS production and $[Ca^{2+}]_i$. It is commonly accepted that a marked increase in $[Ca^{2+}]_i$ can lead to ROS production (Chakraborti et al., 1999). In our model, however, when $[Ca^{2+}]_i$ is kept around 300 nM no ROS production was detected, but a reduction of $[Ca^{2+}]_i$ to around 50 nM induces a delayed significant ROS increase. Besides, during ROS production, $[Ca^{2+}]_i$ levels are not changed before, during or posterior to ROS increase. Therefore ROS production, particularly that related to cell death, is not affecting $[Ca^{2+}]_i$ levels. These results then support the notion that, in our model, ROS generation is mediated by a cellular mechanism, which is independent of an increase of $[Ca^{2+}]_i$ that remains to be elucidated. On the other hand, the protective effect of antioxidants observed in this study was not mediated by a modification of cytosolic Ca^{2+} levels. Thus, although Ca^{2+} levels seem to be the initial signal that directly or indirectly leads to ROS production and cell death, the requirement to induce CGN death is an increase in ROS levels, regardless of the $[Ca^{2+}]_i$. As it was already mentioned, this is supported by the fact that in CGN maintained in 300 nM $[Ca^{2+}]_i$ apoptotic events and CGN death were directly induced by increasing ROS with XA/XO treatment.

As others and we have reported, the activity of caspase-3 is induced by a reduction in $[Ca^{2+}]_i$ and both events could be related to apoptotic cell death of CGN (Morán et al., 1999). In the present study we found that all antioxidants tested inhibited partially both the activity and processing of caspase-3 and that this inhibition was not

mediated by any alteration in $[Ca^{2+}]_i$. The inhibition by antioxidants of caspase-3 activity did not correspond in magnitude to the observed inhibition of its processing. This suggests that antioxidants could act at different levels like processing of the proenzyme or the activity. It remains to be elucidated whether this inhibitory action could also affect the expression and levels of the mRNA, which is known to be markedly increased by K5 in CGN (Morán et al., 1999). Also, on the other hand, an interesting observation was that although there was a complete inhibition by the antioxidants tested on PS translocation, ROS production and caspase-8 activity; the effect of these antioxidants was only partial for cell death and caspase-3 activity and processing. One possible explanation for this result is that a component of caspase-3 activation is not related to oxidative stress. Alternatively, it could be possible that other initiator caspases besides caspase-8 involved in caspase-3 activation could be differentially affected by these antioxidants.

In conclusion, we have shown that CGN apoptotic death induced by low potassium involves the activation of caspase-8 and caspase-3, but not caspase-9 or caspase-1. CGN death is triggered by a $[Ca^{2+}]_i$ reduction, which is followed by increases in ROS levels. Once $[Ca^{2+}]_i$ reduction initiates the process of cell death, however, the ROS production seems to a the critical event independent on $[Ca^{2+}]_i$. The generation of ROS occurred at three different times, being those produced after 3–4 hr involved in the process of cell death. Caspases activation and PS translocation were downstream of ROS production. These findings indicate that ROS play a critical role in the initiation of the apoptotic process including the activation of caspases.

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