Protection against apoptosis by monoamine oxidase A inhibitors

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Abstract Several lines of evidence have been accumulating indicating that an important role may be played by mitochondrial homeostasis in the initiation phase, the first stage of apoptosis. This work describes the results obtained by using different inhibitors of monoamine oxidases (MAO), i.e. pargyline, clorgyline and deprenyl, on mitochondrial integrity and apoptosis. Both pargyline and clorgyline are capable of protecting cells from apoptosis induced by serum starvation while deprenyl is ineffective. These data represent the first demonstration that MAO-A inhibitors may protect cells from apoptosis through a mechanism involving the maintenance of mitochondrial homeostasis.
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Key words: Apoptosis; Mitochondrial membrane potential; Amine oxidase

1. Introduction

Apoptotic cell death is well known to be characterized by several subcellular alterations leading to a specific cell death program [1]. Three steps can be distinguished: the initiation, effector and degradation phases. In the first phase, the cell receives the death stimulus which may be represented by exogenous physical, chemical or biological agents or by a lack of intracellular survival factors. In the subsequent effector phase several reactions can lead to cell death triggering. This regulatory step is followed by the degradation phase which is irreversible and characterized by typical morphological and histochemical markers of apoptosis including DNA fragmentation [2]. It has recently been hypothesized that mitochondrial membrane potential [$\Delta\Psi_m$] may play a key role in the early events of the apoptotic cascade [3]. Thus, regulatory mechanisms underlying mitochondrial activity in the cell death program are currently under investigation in several laboratories. Some endogenous and exogenous compounds which could exert a proapoptotic or antiapoptotic regulatory role, influencing mitochondrial homeostasis, have been studied [3–5]. The presence of monoamine oxidase (MAO) on the outer mitochondrial membrane can generate cytotoxic species involved in the alteration processes of subcellular structures. For instance, the damage of mitochondrial DNA due to hydrogen peroxide, produced by oxidative deamination of tyramine by FAD-dependent monoamine oxidase, has been examined [6]. In light of this, we investigated the activity of inhibitors of different MAO isoforms (A and B) on either the

mitochondrial membrane potential or apoptosis. Analytical cytology analyses revealed that maintenance of the mitochondrial homeostasis by pargyline and clorgyline is associated with a partial hindering of the apoptotic process.

2. Materials and methods

2.1. Cell cultures

Human melanoma cells (M14) were grown in monolayer in modified RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, 1% non-essential amino acids, 5 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in an incubator at 37°C containing a 95% air/5% CO_2 atmosphere.

2.2. Withdrawal of growth factors (serum starvation) and treatments with MAO inhibitors

35×10^4 cells/ml were plated in the medium supplemented with 10% FCS. After 24 h the medium was replaced with RPMI 1640 without serum, or containing 1% or 10% FCS in the presence of different concentrations (10^{-3}; 10^{-5}; 10^{-7} and 10^{-9} M) of the following drugs. (a) Pargyline (Sigma-Aldrich, N-benzyl-N-methyl propargylamine, K_i 15.3 μM for MAO-A and 1.8 μM for MAO-B). High concentrations of pargyline inhibit both MAO isoforms while low concentrations inhibit only MAO-B [7]. (b) Clorgyline (Sigma-Aldrich, N-methyl-N-propargyl-3-(2,4-dichlorophenoxy)-propylamine). This compound is a specific MAO-A inhibitor. (c) Deprenyl (ICN Biomedicals, (R)-N,α-dimethyl-N-2-propynylbenzeneethaneamine hydrochloride). This compound is a specific inhibitor of MAO-B. Over the course of 48 h experiments these drugs were administered twice (time O and 24 h). After 24 and 48 h at 37°C, cells were collected for apoptosis evaluation and measurement of mitochondrial membrane potential and MAO activity.

2.3. Apoptosis evaluation

In order to determine apoptosis, the chromatin dye Hoechst 33258 (Molecular Probes, Eugene, OR, USA) was used to evaluate DNA fragmentation as previously described [8]. The samples were observed with a Nikon Microphot fluorescence microscope and quantitative evaluation of apoptotic cells was performed by counting at least 300 cells at 500× magnification.

2.4. Mitochondrial membrane potential

Cossarizza and coworkers have recently set up a new assay for measuring mitochondrial membrane potential ($\Delta\Psi_m$) and to study apoptosis [9]. The method uses the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide (JC-1, Molecular Probes). This probe, a monomeric molecule emitting at 527 nm after excitation at 490 nm, is capable of selectively entering the mitochondria. However, JC-1 is able to form aggregates associated with a large shift in emission (590 nm) depending on the $\Delta\Psi_m$. Cell suspension was adjusted to a density of 0.5×10^6 cells/ml, kept in the dark for 10 min at room temperature in medium containing 10% FCS and 10 μg/ml JC-1 probe. JC-1 was dissolved and stored according to the manufacturer's instructions. After treatment, cells were washed twice with PBS by centrifugation ($1000 \times g$, 5 min) and then resuspended in 400 μl PBS. Samples containing no fewer than 2×10^5 cells were analyzed by FACScan flow cytometer (Becton Dickinson, San José, USA) equipped with a single 488 nm argon laser.

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Data were collected in list mode and analyzed using Lysys II software (Becton Dickinson). Mitochondrial $\Delta\Psi$ alterations were analyzed in cells undergoing apoptosis and in cells treated with MAO inhibitors as described above.

2.5. Flow cytometry measurements

The filter in front of the FL1 photomultiplier (PMT) has a bandwidth of 30 nm and transmits at 530 nm, while the filter used in the FL2 channel has a bandwidth of 42 nm and transmits at 585 nm. For the analysis of cells stained with JC-1, the PMT value of the FL1 detector was set at 360 V, and FL2 PMT at 300 V; FL1-FL2 and FL2-FL1 compensation were 5.0% and 10.0% respectively.

2.6. MAO activity assay

After treatment with different drugs, cells were collected and washed three times by centrifugation ($1000 \times g$, 5 min) with PBS. The cells were resuspended in PBS to the appropriate concentration (50×10^6 cells/ml) and MAO-A and -B activity was assayed fluorimetrically using kynuramine as substrate in the presence or absence of various inhibitors. The activity was determined by measuring 4-hydroxyquinoline (4HQ) and expressed per mg of protein during 30 min of incubation, at λ_{exc} 315 nm and λ_{em} 390 nm [10]. Endocellular substrates of MAO, serotonin and dopamine, were detected by HPLC method, according to Matés et al. [11].

2.7. Statistical analysis

The values reported in the figures are the means ± standard deviation (S.D.) from three separate experiments. Statistical analyses were performed using Student's t-test. A P value lower than 0.05 was considered significant.

Fig. 2. Apoptosis rates in M14 cells grown in serum-deprived medium and treated with different concentrations of the MAO inhibitors pargyline, clorgyline or deprenyl for 48 h. The apoptotic phenomenon is significantly decreased only by administration of pargyline and clorgyline.

3. Results

3.1. Apoptotic evaluation

Two different experimental conditions were considered: M14 cells maintained in culture medium containing a low amount of serum (1%) and M14 cells in medium deprived of FCS. M14 cells grown in RPMI 1640 supplemented with 10% FCS were used as controls. In these conditions, after 24 h, the levels of apoptosis in control cells, detected as described in Section 2, were very low ($< 10\%$). As expected, some isolated apoptotic cells along with numerous mitotic figures were found (not shown). By contrast (Fig. 1), after serum starvation, the apoptosis rate was significantly higher and high pargyline concentrations (1 mM), capable of inhibiting both MAO-A and MAO-B activity, were able to significantly reduce these values. In particular, after 48 h starvation a remarkable apoptotic induction was detected: partial or complete serum starvation led in the supernatants to the apoptosis rates shown in Fig. 1 (mean values were 28% and 57% respectively). Treatments with pargyline were able to partially prevent this process by about 50% in both experimental conditions ($P < 0.01$). Fig. 1b,c shows the morphological features of apoptotic cells after 48 h starvation (b) and after pargyline administration to starved cultures (c). A quantitative evaluation of detached cells flowing freely in the supernatants also indicated that the presence of pargyline in the medium was capable of partially impairing cell detachment from the substrate. In particular, a significant decrease in the number of cells in the supernatants was observed in the presence of pargyline ($44.2 \pm 8\%$ after 48 h serum starvation, $21.6 \pm 5\%$ in pargyline treated samples, $P < 0.05$). Moreover, the above results were also confirmed by MAO activity in the cells. In fact, the cells incubated for 48 h in the absence of serum showed an inhibition of about 80% of the MAO activity in the presence of 1 mM pargyline (4HQ values from 50 pmol/mg protein normally detectable in starved cells decreased to 8.8 pmol/mg protein in 10^{-3} M pargyline treated cells). The inhibitory effect on MAO activity was also confirmed by an increase of serotonin (0.59–4.41 nmol/10^6 cells) and dopamine (0.012–0.24 nmol/10^6 cells). Further analyses of apoptotic cells were also conducted by using lower pargyline concentrations in an attempt to inhibit only the MAO-B enzyme. Results are summarized in Fig. 2 and indicate that lower pargyline concentrations (10^{-5} and 10^{-7} M) correspond to lower protection from apoptosis. The lowest dose used (10^{-9} M) was com-
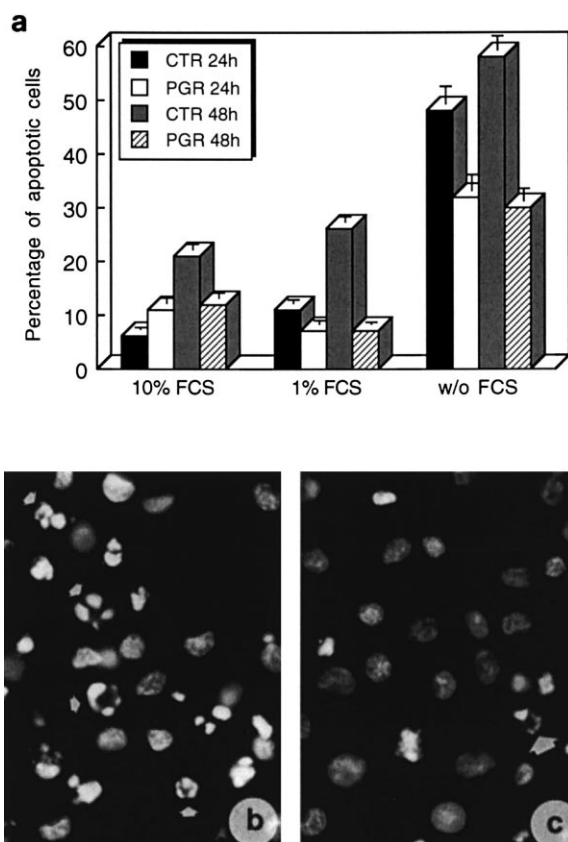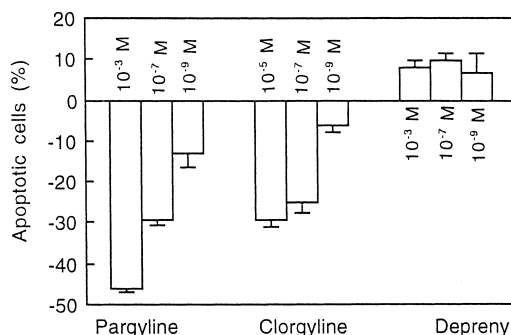
Fig. 1. Apoptosis rate after 24 and 48 h of cell treatment with pargyline. Two different experimental conditions were considered: M14 cells maintained with low levels of serum (1%) and M14 cells completely deprived of FCS. M14 cells growing in 10% FCS supplemented medium were used as controls. b,c: Morphological features of apoptotic cells after 48 h starvation (b) and after pargyline administration to starved cultures (c). Arrows indicate chromatin condensation and/or clumping typical of apoptosis.
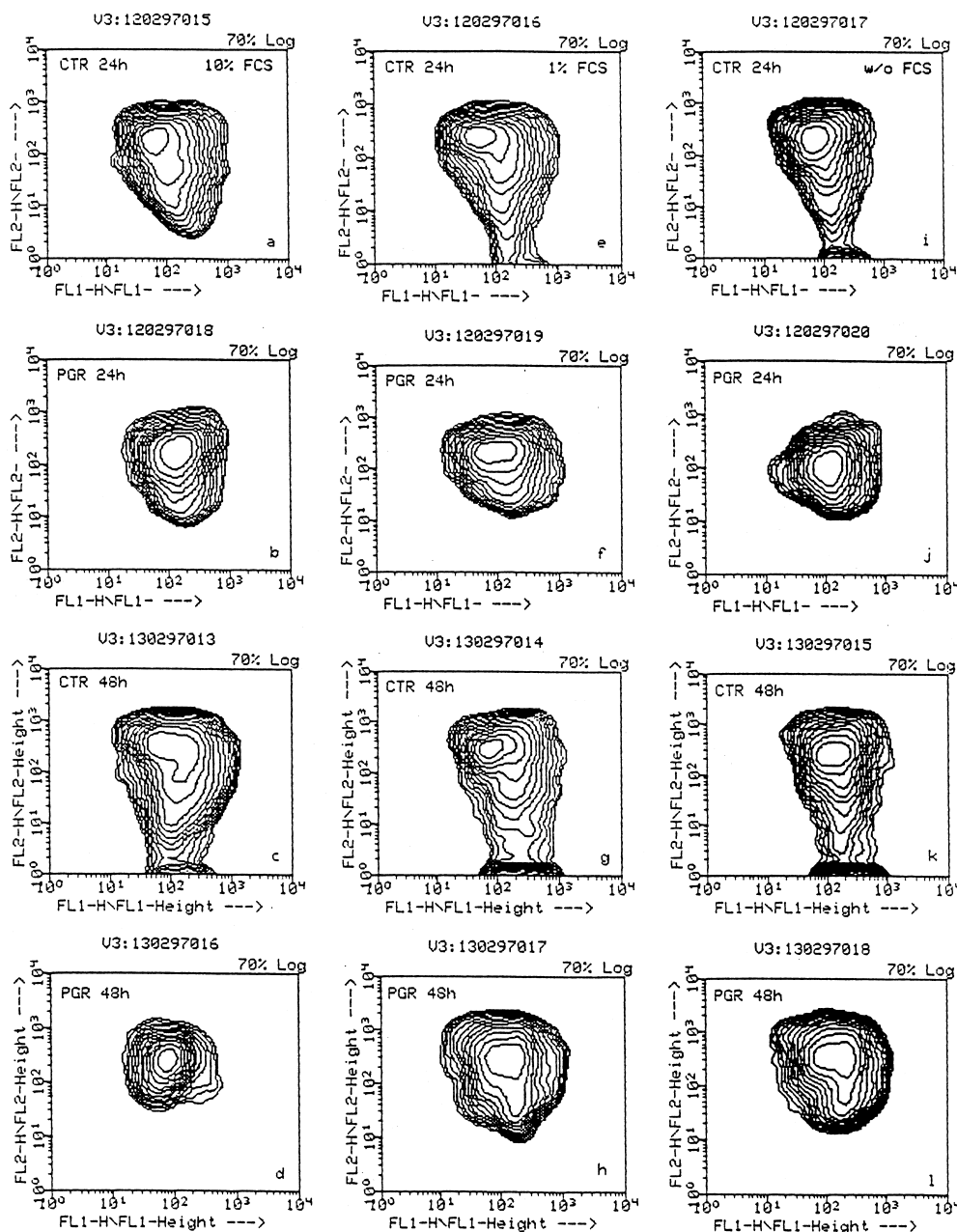
Fig. 3. Mitochondrial membrane potential ($\Delta\Psi_m$). Left panel (a–d): 10% FCS-supplemented cells (controls); central panel (e–h): 1% FCS-supplemented cells; right panel (i–l): starved cultured cells. Abscissa: FL1 (green fluorescence, log scale); ordinate: FL2 (orange fluorescence, log scale). CTR: pargyline untreated cells; PGR: pargyline treated. A representative experiment of three is shown.

pletely ineffective (Fig. 2) and 4HQ values were at least double those reported above for pargyline 1 mM (e.g. 16 pmol/mg protein in 10^{-7} M pargyline treated samples). In addition, parallel experiments carried out by using specific inhibitors of MAO-A (clorgyline) and MAO-B (deprenyl) were also performed. According to the above results, a concentration-dependent protection was found after clorgyline administration while deprenyl was ineffective at all concentrations used (Fig. 2). In particular, high concentrations of clorgyline (10^{-3} M) were toxic to M14 cells (cell loss $> 80\%$) while low concentrations (10^{-5}–10^{-7} M, Fig. 2) were capable of producing a significant decrease of the apoptotic cell rate. Accordingly, 4HQ concentrations were very low (about 7 pmol/mg protein). By contrast, no statistically significant effects were found

after deprenyl treatments either in the apoptotic values or in the 4HQ concentrations, which were similar to those of starved cells, 41 pmol/mg protein.

3.2. Mitochondrial membrane potential evaluation

Cell loading with JC-1 was then performed in order to obtain information about $\Delta\Psi_m$ in both control and treated cells. Cytofluorimetric studies allowed us to detect that $\Delta\Psi_m$ was affected by serum deprivation in a time dependent manner as shown in Fig. 3. After 48 h starvation M14 cells showed a marked increase in mitochondrial membrane depolarization. This is represented by the alterations of the contour plot as shown in Fig. 3k. In fact, according to the above method [8], the cells with depolarized mitochondria are those
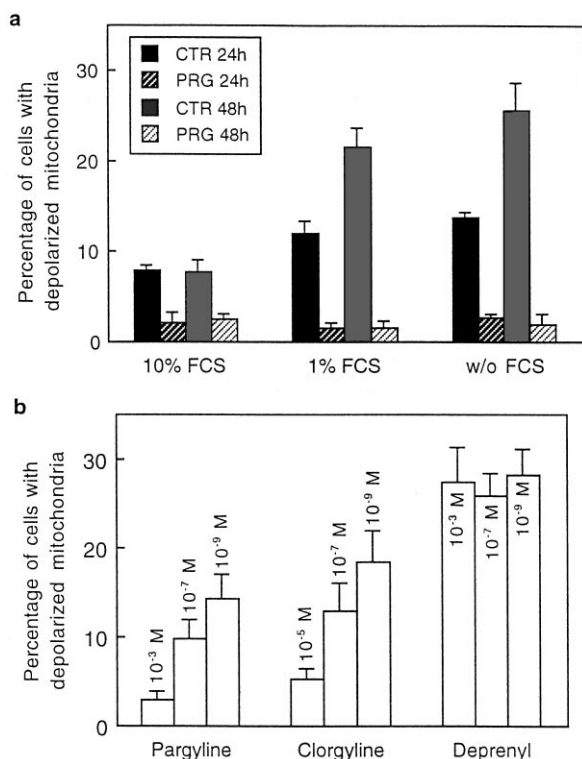
Fig. 4. Percentage of M14 cells with depolarized mitochondria. a: Deprivation of serum induced a marked increase in the number of cells with mitochondrial depolarization. Pargyline treatment (10^{-3} M) is able to significantly prevent ($P < 0.01$) mitochondrial damage. b: Treatment with different concentrations of pargyline, clorgyline or deprenyl. Only treatment with pargyline and clorgyline significantly ($P < 0.01$) prevents apoptosis-associated mitochondrial damage, induced by starvation, in a dose-dependent manner. In contrast, deprenyl is ineffective at all doses used.

moving from the middle of the quadrant to the lower right corner, as they lose greenish orange fluorescence (in FL2). This effect was consistently reduced by the presence of pargyline in the medium (Fig. 3l). In Fig. 4a a quantitative evaluation of these changes is shown. A high percentage of cells with depolarized mitochondria is detectable after serum deprivation, while the number of cells with compromised mitochondria showed a significant decrease after pargyline administration ($P < 0.01$). The same analyses have also been carried out by analyzing mitochondrial membrane potential after treatment with various concentrations of MAO inhibitors. The results obtained indicate that, according to the above reported results on apoptosis (Fig. 2), both pargyline and clorgyline, but not deprenyl, are capable of protecting the cells against mitochondrial damage. In fact, flow cytometry analyses indicated that significant protection is offered by these drugs after both partial (1%) and complete starvation protocols (not shown). The quantitative evaluation of these data is shown in Fig. 4b and indicates significant protection exerted only by drugs acting on MAO-A, i.e. high pargyline concentrations and clorgyline.

4. Discussion

The importance of mitochondrial activity in the first phase of apoptotic triggering was first pointed out in 1993 [5]. In particular, mitochondrial membrane potential was investi-

gated in order to determine whether any alteration of this important function could be related to the cascade of events occurring intracellularly during apoptosis [2]. Different approaches have been considered, including structural and biophysical ones [12,13]. However, efforts made by Kroemer's group using a plethora of different fluorescent probes disclosed the possibility of approaching the study of mitochondrial activity in the course of apoptosis by flow cytometry [3]. Our group also contributed to these efforts by proposing the use of a specific probe, JC-1, which is endowed with a mitochondrial targeted compound for analyzing alterations of mitochondrial membrane potential [9,14,15]. The present work describes one early event in the induction of apoptosis, which may be represented by the alteration of mitochondrial integrity and its homeostasis. Pargyline and clorgyline were able to prevent this process through a mechanism involving the maintenance of mitochondrial homeostasis. This may be related to the previously suggested activity of the drugs on ion regulation [16] as well as to a decreased production of reactive oxygen species. MAOs, as reported above, catalyze the oxidative deamination of biogenic amines producing aldehydes and H_2O_2 which, in turn, are key molecules in apoptotic induction mechanisms [17]. MAO inhibition is in fact associated with a decrease in production of both oxygen radicals and aldehydes during the catalytic cycle representing a risk for mitochondrial integrity [6]. For instance, it has been shown that H_2O_2 and aldehydes produced during the enzymatic oxidation of exogenous spermine by purified bovine serum amine oxidase have been shown to cause cytotoxicity in cultured cells [18]. Further, oxidation-dependent mechanisms of apoptosis after exposure to spermidine have also been described [19].

Mitochondrial MAO-A and -B are irreversibly and specifically inhibited by propargylamine (or acetylenic) derivatives [20]. In particular, pargyline is capable of inhibiting both MAO isoforms when used at high (millimolar) concentrations [20]. In fact, in our experimental conditions, a powerful inhibitory effect on both apoptosis and MAO activity was exerted by high doses of pargyline. In contrast, with lower drug concentrations (beyond 10^{-7} M), mitochondrial membrane potential and apoptotic cell death appeared to occur normally. In this context, the importance of other specific MAO inhibitors, such as clorgyline for MAO-A and deprenyl for MAO-B, was considered. We found that only the former was effective in providing a significant protection from apoptosis and $\Delta\Psi$ changes. The absence of deprenyl protection could be imputable to the low MAO-B content in the melanoma cells considered here. On the other hand, the non-linear inhibition of MAO activity as a function of pargyline and clorgyline concentrations could be partially explained by the fact that these drugs are suicide inhibitors [21] and by the observation that a non-specific binding of MAO inhibitors to sites other than MAO active sites has been proposed [22]. These data seem to imply that, at least in withdrawal of growth factor-induced apoptosis, i.e. in protein synthesis-independent apoptosis, an important role may be played by MAO-A-associated mitochondrial activity. In fact, a different mechanism for protecting from apoptosis in pheochromocytoma cells (PC12) using deprenyl was reported [23]. Induction of protein synthesis may explain the protective effect of low deprenyl concentrations (10^{-9}–10^{-11} M), which are not sufficient to inhibit MAO-A, the only isoform present in PC12 cells; partial apoptosis protection in the same cells by pargy-

line (10^{-3}–10^{-7} M) was also observed [23]. Thus, in conclusion, our work offers the first evidence of a possible specific activity of MAO-A inhibitors in partially hindering the mitochondrially triggered cell death program and, more in general, in the maintenance of mitochondrial homeostasis. Our conclusions are in agreement with the recent paper by Hu and Pegg [24] on the apoptosis hindering exerted by polyamine oxidase activity inhibitors. These authors suggest that programmed cell death can be caused by the oxidation products of polyamines. In light of this, our data further address the question strongly suggesting that the mitochondrial H_2O_2 and/or aldehyde in situ production can also play a major role in triggering apoptosis.

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