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Oxidation products of polyamines induce mitochondrial uncoupling and cytochrome *c* release

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Abstract Spermine is shown to uncouple isolated mitochondria and to trigger the selective release of cytochrome *c*. Pargyline, an inhibitor of amine oxidase (AO), fully prevented these effects of spermine, which instead were potentiated by exogenous AO. Hydrogen peroxide, an oxidation product of spermine, mimicked the effects of spermine on mitochondria, while the addition of catalase prevented them. Spermidine and putrescine also caused mitochondrial uncoupling and triggered cytochrome *c* release, with a potency which correlated with the substrate preference of mitochondrial AO. Pargyline protected human lymphoma U937 cells against UVB-induced apoptosis, by reducing AO activity, mitochondrial uncoupling and cytochrome *c* release. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Amine oxidase; Apoptosis; Cytochrome *c*; Hydrogen peroxide; Mitochondrial potential; Polyamine

1. Introduction

The polyamines spermine and spermidine are synthesized in all eukaryotic cells starting from putrescine, and are thought to be involved in a variety of cellular processes, including the control of cell growth [1,2]. Recently, intracellular accumulation of polyamines has been shown to induce programmed cell death (PCD, or apoptosis) in various cell types [3–5], through activation of the caspase cascade [6–8]. The activation of caspases is triggered by release of mitochondrial cytochrome *c* [9–11], which has been recently shown to be induced by spermine and spermidine [12]. However, the mechanism of this effect of polyamines has not yet been elucidated. On the other hand, it has been demonstrated that exogenous spermine becomes cytotoxic in the presence of amine oxidase (AO) in the extracellular space [13,14], suggesting that the oxidation products of polyamines, rather than polyamines themselves, are cytotoxic to the mammalian cells. AOs catalyze the oxidative deamination of biologically important amines, with the formation of the corresponding aldehyde, hydrogen peroxide and ammonia [15]. Two isoforms, termed monoamine oxidases A and B, are present in approximately the same amounts (40% and 60%, respectively) in the outer mitochondrial membrane [16], and inhibition of either of them has been shown to protect cells against apoptosis [17,18]. Here, we used a cell-free mitochondrial preparation to investigate whether natural polyamines might trigger cytochrome *c* release through AO-catalyzed oxidation, and whether this process is linked to disruption of mitochondrial integrity. Moreover, we used a cellular model of apoptosis dependent on caspase activation, i.e. UVB irradiation [19], in order to assess the possible role of AO activity in a physiologically relevant model of PCD [19,20].

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Abbreviations: AO, amine oxidase; BSA, bovine serum albumin; CCCP, *m*-chlorophenylhydrazone; $\Delta\Psi$, membrane potential; ELISA, enzyme-linked immunosorbent assay; GAM-AP, goat anti-mouse alkaline phosphatase conjugates; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide; MIB, mitochondria incubation buffer; MPT, mitochondrial permeability transition; PBS, phosphate-buffered saline; PCD, programmed cell death

2. Materials and methods

2.1. Materials

Chemicals were of the purest analytical grade. Percoll, spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine), spermidine (*N*-[3-aminopropyl]-1,4-butanediamine), putrescine (1,4-diaminobutane), hydrogen peroxide, pargyline (*N*-benzyl-*N*-methyl propargylamine), catalase (from bovine liver; activity = 5000 U/mg protein, 1 U decomposing 1.0 μmol H_2O_2 per min, at pH 7.0 and 25°C), AO (from bovine plasma; activity = 100 U/g, 1 U oxidizing 1.0 μmol benzylamine per min, at pH 7.4 and 25°C), *m*-chlorophenylhydrazone (CCCP), cytochrome *c* (from rat heart), and cyclosporin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR, USA). [1,4-^{14}C]Putrescine dihydrochloride (10 Ci/mmol = 370 GBq/mmol) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Anti-cytochrome *c* monoclonal antibodies were purchased from PharMingen (San Diego, CA, USA) and goat anti-mouse alkaline phosphatase conjugates (GAM-AP) were from Bio-Rad (Richmond, CA, USA). Non-immune mouse serum was from Nordic Immunology (Tilburg, The Netherlands).

2.2. Preparation of mitochondria

Mitochondria were prepared from the liver of female Wistar rats weighing about 350 g by standard differential centrifugation, followed by Percoll purification [12]. The livers were homogenized gently with an UltraTurrax apparatus in 3 vol (w/v) of 0.5% bovine serum albumin (BSA) containing 180 mM KCl and 10 mM EDTA (pH 7.2). The homogenate was centrifuged for 5 min at 1000×*g*, and the supernatant was filtered and centrifuged for 10 min at 8000×*g*. The pellet was resuspended in 0.5% BSA containing 180 mM KCl and 10 mM EDTA (pH 7.2) and centrifuged at 5000×*g* for 5 min. The final pellet was gently resuspended in 0.5 ml of mitochondria incubation buffer (MIB), consisting of 0.25 M sucrose, 1 mM KH_2PO_4 and 10 mM HEPES (pH 7.4). This crude mitochondria preparation (0.5 ml) was layered on top of 15 ml of a solution consisting of 30% Percoll, 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.4). Self-generating Percoll gradient was developed by centrifugation at 35 000×*g* for 30 min at 4°C. The mitochondrial band was separated from less dense

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contaminants and broken mitochondria, was collected with a Pasteur pipette and was washed twice in 5 ml MIB by centrifuging for 5 min at 8000×*g*. The final pellet was resuspended in MIB at a protein concentration of 10 mg/ml, and was used as such. Mitochondria isolated by this procedure were ~90% intact by cytofluorimetric analysis in a FACScalibur Flow Cytometer (Becton Dickinson, Bedford, MA, USA) [21], corroborating previous functional data on a preparation obtained with the same method [12].

2.3. Cell culture, irradiation and evaluation of apoptosis

Human lymphoma U937 cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 25 mM HEPES, 2.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum [22]. UVB irradiation was performed as described [19,20]. The cells (5×10^6/test) were washed twice in pre-warmed phosphate-buffered saline (PBS) and were irradiated in PBS in a 10 cm Petri dish, using a UVB lamp at a dose of 77 mJ/cm^2 [20]. In pilot experiments, this irradiative dose was found to induce apoptosis in a large number of cells. The effect of various compounds was tested by adding each substance to PBS immediately before UVB irradiation. Control cells were treated in the same way but UVB irradiation.

Evaluation of apoptosis was performed by the cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany), based on the evaluation of DNA fragmentation by an immunoassay for histone-associated DNA fragments in the cell cytoplasm [19]. Cell death was evaluated 24 h after exposure of cells to UVB. The ELISA test was validated by cytofluorimetric analysis in a FACScalibur Flow Cytometer, with control U937 cells containing 4 ± 1 apoptotic bodies every 100 cells analyzed [22].

2.4. Analysis of mitochondrial uncoupling

Mitochondrial uncoupling was measured using the fluorescent probe JC-1, as described [23]. JC-1 (dissolved in dimethylsulfoxide) was used at 20 μM final concentration, treating the controls with vehicle alone (1% of the final volume). After the treatment, isolated mitochondria or U937 cells were washed in PBS, incubated for 20 min at 37°C in PBS and then analyzed in a FL1/FL2 dot plot (530 nm/570 nm) by a FACScalibur Flow Cytometer.

2.5. Determination of cytochrome c release

30 min after each treatment, isolated mitochondria or U937 cells were centrifuged at 8000×*g* for 10 min and supernatants (25 μg protein/lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS–PAGE (12%) under reducing conditions, followed by electroblotting onto 0.45 μm nitrocellulose filters (Bio-Rad), as reported [22]. Prestained molecular weight markers (Bio-Rad) were BSA (80 kDa), ovalbumin (50 kDa), carbonic anhydrase (34 kDa) and lysozyme (22 kDa). Immunodetection of cytochrome *c* on nitrocellulose filters was performed with specific anti-cytochrome *c* monoclonal antibodies, diluted 1:250. GAM-AP were used as secondary antibody at 1:2000 dilution. The amount of cytochrome *c* released from isolated mitochondria or into the cytosol of U937 cells under the same experimental conditions was quantified by ELISA, as reported [22]. Supernatants (25 μg protein/well) were reacted with anti-cytochrome *c* monoclonal antibodies, diluted 1:250, using GAM-AP as secondary antibody at 1:2000 dilution. Color development of the alkaline phosphatase reaction was recorded at 405 nm, using *p*-nitrophenylphosphate as substrate. The absorbance values of the unknown samples were within the linearity range of the ELISA test, assessed by calibration curves with known amounts of cytochrome *c* (in the range 0–200 ng/well) [22].

2.6. Assay of amine oxidase

The activity of AO (amine:oxygen oxidoreductase [deaminating], EC 1.4.3.4) in isolated mitochondria was determined at 25°C in 0.1 M potassium phosphate buffer (pH 7.4), by measuring the consumption of oxygen in air-saturated solutions (i.e. 240 μM O_2) by a YSI-5301 oxygen monitor (Yellow Springs Instrument, Yellow Springs, OH, USA), equipped with a Clark electrode [15]. AO activity in U937 cells was determined by measuring the formation of [^{14}C]Δ^1-pyrroline from [^{14}C]putrescine as described [24]. The incubation mixture contained in 0.1 M potassium phosphate (pH 7.4) the cell homogenate (100 μg protein/test), and 1.5 mM putrescine containing 0.5 μCi [^{14}C]putrescine. After 2 h at 37°C, the reaction was stopped by adding 200 mg of $NaHCO_3$ and 10 ml toluene, and product formation was determined by liquid scintillation counting in a LKB1214 Rackbeta spectrometer (Amersham Pharmacia Biotech).

2.7. Statistical analysis

Data reported in this paper are the mean (± S.D.) of at least three independent determinations, each performed in duplicate. Statistical

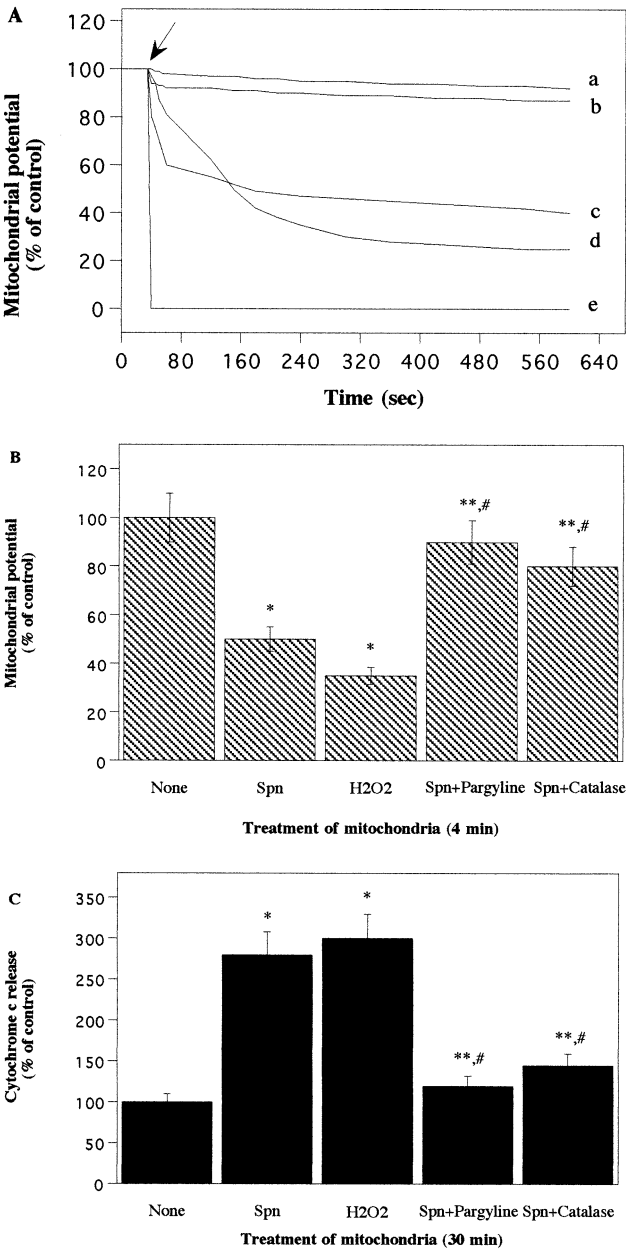

Fig. 1. A: Mitochondrial potential of isolated mitochondria, untreated (trace a) or treated with 100 μM spermine+1 mM pargyline (b), 100 μM spermine (c), 20 μM H_2O_2 (d), 50 μM CCCP (e). Treatment of mitochondria with 100 μM spermine+50 U catalase yielded a trace superimposable to trace b, and was omitted for the sake of clarity. Arrow indicates the addition of the various compounds to mitochondrial suspensions. B: Mitochondrial uncoupling, calculated 4 min after the same treatments, and from similar traces, as in panel A. C: Release of cytochrome *c* from isolated mitochondria 30 min after the same treatments as in A (100% = 0.150 ± 0.015 absorbance units at 405 nm). In B and C, * denotes $P<0.01$ compared with control; ** denotes $P>0.05$ compared with control; # denotes $P<0.01$ compared with spermine-treated samples. Spn, spermine.

analysis was performed by the Student's *t*-test, elaborating experimental data by means of the InStat program (GraphPAD Software for Science, San Diego, CA, USA).

3. Results

Intact isolated mitochondria showed a membrane potential ($\Delta\Psi$), which was immediately dissipated by addition of 50 μM CCCP (Fig. 1A), a powerful uncoupling agent [9]. The natural polyamine spermine also abolished mitochondrial $\Delta\Psi$ in a dose-dependent way, with a maximum at 100 μM (Fig. 1A and data not shown). At this concentration, spermine reduced $\Delta\Psi$ to 50% of the untreated control within 4 min (Fig. 1A and B). Coincubation of mitochondria with 1 mM pargyline, an inhibitor of AO activity [17], fully inhibited the uncoupling effect of 100 μM spermine, much alike the coincubation with 50 U catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) (Fig. 1A and B). The effect of 100 μM spermine on $\Delta\Psi$ was mimicked by 20 μM H_2O_2, or by 50 μM spermine in the presence of 2 U exogenous AO (Fig. 1A and B and data not shown). Cyclosporin A, a selective inhibitor of the mitochondrial permeability transition (MPT) pores [10], was ineffective at 10 μM. Dissipation of mitochondrial $\Delta\Psi$ by spermine was paralleled by a dose-dependent release of cytochrome *c* (Fig. 2). This leakage reached a maximum at 100 μM spermine and, most notably, was selective; indeed, cytochrome *c* was the only protein detectable by Western blot in supernatants from isolated mitochondria (Fig. 2). Spermine (100 μM) caused a 2.8-fold increase in cytochrome *c* release, corresponding to approximately 3 μg/mg of protein according to calibration curves with pure cytochrome *c* [21]. Coincubation of isolated mitochondria with 1 mM pargyline or 50 U catalase fully prevented cytochrome *c* release induced by 100 μM spermine, while 20 μM H_2O_2 similarly induced it (Fig. 1C). Under the same experimental conditions, spermidine and putrescine, both used at 100 μM, caused a mitochondrial uncoupling of 60 and 70%, and a cytochrome *c* release of 250 and 200% of the untreated controls, respectively (Table 1). Remarkably, the order of efficacy, spermine > spermidine > putrescine, correlated with the substrate preference of mitochondrial AO, which oxidized spermine, spermidine and putrescine at a rate of 200 ± 20, 170 ± 17 and 120 ± 12 nmol min^{-1} mg $protein^{-1}$ (Table 1).

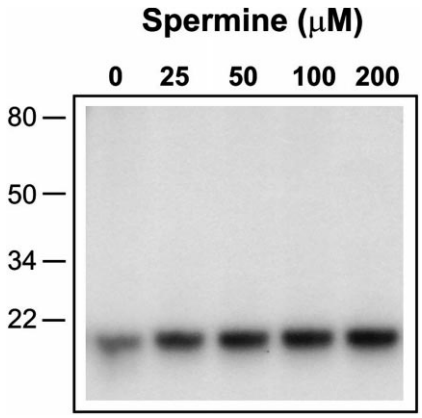

Fig. 2. Western blot analysis of cytochrome *c* in supernatants (25 μg/lane) of isolated mitochondria, reacted with specific anti-cytochrome *c* monoclonal antibodies. Molecular weight markers are shown on the left-hand side.

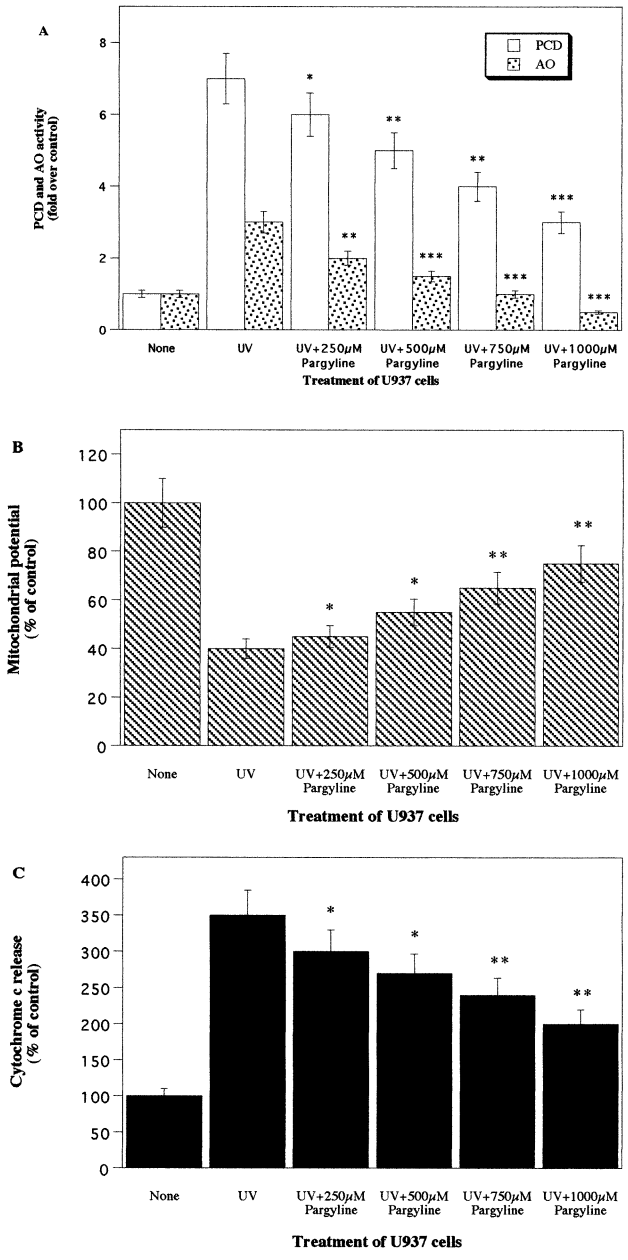

Fig. 3. A: Induction of PCD and AO activity in U937 cells irradiated with UVB light, in the presence of different amounts of pargyline (100% AO activity = 30 ± 3 pmol min^{-1} mg $protein^{-1}$). B: Mitochondrial potential of U937 cells treated as in A. C: Cytochrome *c* release in the cytosol of U937 cells treated as in A (100% = 0.120 ± 0.012 absorbance units at 405 nm). In all panels, * denotes $P > 0.05$ compared with UV-treated cells; ** denotes $P < 0.05$ compared with UV-treated cells; *** denotes $P < 0.01$ compared with UV-treated cells.

Irradiation of human lymphoma U937 cells with UVB light induced a 7-fold increase in apoptosis (Fig. 3A), corresponding to approximately 28 apoptotic bodies every 100 cells analyzed [21]. The increase in PCD was paralleled by a 3-fold increase in AO activity, from 30 ± 3 to 90 ± 9 pmol min^{-1} mg $protein^{-1}$, and pargyline (in the range 0–1 mM) dose-dependently inhibited both PCD and AO activity induced by UVB irradiation (Fig. 3A). In the presence of 1 mM pargyline, PCD and AO activity were reduced to approximately 40 and 15%, respectively, of the values observed in irradiated cells (Fig.

Table 1
Effect of polyamines on $\Delta\Psi$, cytochrome *c* release and AO activity in isolated mitochondria

Polyamine (100 μM)	Membrane potential (% of control)	Cytochrome *c* release (% of control)	AO activity (nmol min^{-1} mg $protein^{-1}$)
None	100	100	N.D.
Spermine	50 ± 5[a]	280 ± 28[b]	200 ± 20
Spermidine	60 ± 6	250 ± 25	170 ± 17
Putrescine	70 ± 7	200 ± 20	120 ± 12

N.D., not determined.
[a]Data from Fig. 1B.
[b]Data from Fig. 1C.

3A). UVB light also reduced mitochondrial $\Delta\Psi$ in U937 cells to 40% of the controls (Fig. 3B), and this was paralleled by an increase in cytochrome *c* release up to 350% of the controls (Fig. 3C). Pargyline (in the range 0–1 mM) dose-dependently prevented mitochondrial uncoupling and cytochrome *c* release induced by UVB, which reached approximately 190 and 60%, respectively, of the values of irradiated cells in the presence of 1 mM inhibitor (Fig. 3C).

4. Discussion

The interaction of polyamines with mitochondria, known for years, generally leads to protection of these organelles against damage. For instance, polyamines regulate calcium transport, ATP synthesis and glutathione extrusion ([12] and references therein), and more generally protect DNA [25] and membrane lipids [26] against oxidative stress. Recently, polyamines have been demonstrated to trigger cytochrome *c* release from heart mitochondria, at the same concentrations used here and with a similar order of potency. However, the underlying mechanism was unclear [12]. Here, we report that spermine-induced cytochrome *c* release from mitochondria was paralleled by dissipation of $\Delta\Psi$, and thus disruption of mitochondrial membrane integrity. We also show that both these effects of spermine were prevented by pargyline (Fig. 1), an inhibitor of AO at millimolar concentrations [17]. Furthermore, addition of exogenous AO potentiated the effect of spermine, and the ability of spermine, spermidine and putrescine to uncouple mitochondria and to trigger cytochrome *c* release correlated with the substrate preference of mitochondrial AO activity (Table 1). Taken together, these data suggest that polyamine oxidation products, rather than polyamines themselves, disrupt mitochondrial integrity. In fact H_2O_2, a product of polyamine oxidation by AO, mimicked the effect of spermine on $\Delta\Psi$ reduction and cytochrome *c* release, and catalase was effective in counteracting it (Fig. 1). These findings suggest that H_2O_2 was responsible for at least most of spermine activity, confirming its ability to induce cytochrome *c* release and caspase activation [27], as well as PCD of mammalian cells [28,29]. It is noteworthy that spermine-induced leakage of cytochrome *c* was selective (Fig. 2), whereas a generalized leakage of proteins has been reported when MPT pores are opened [30]. This observation, together with the fact that the MPT inhibitor cyclosporin A was ineffective on spermine-induced dissipation of $\Delta\Psi$, strongly suggests that MPT pores were not involved in this process.

The possible relevance of the activation of AO, and polyamine oxidation thereof, in cellular apoptosis was evaluated by irradiating human lymphoma U937 cells with UVB. Irradiation was chosen as an inducer, because it causes PCD through caspase activation, a process reported also upon treatment of cells with spermine [6,7]. We showed that UVB-induced apoptosis was paralleled by an increase in cellular activity of AO (Fig. 3). Activation of this enzyme was a cause and not an effect of the apoptotic program, because pargyline dose-dependently inhibited UVB-induced PCD (Fig. 3). Interestingly, irradiation led also to mitochondrial uncoupling and cytochrome *c* release, which were significantly ($P < 0.05$) reduced in the presence of pargyline (Fig. 3). Altogether, these findings suggest that oxidation of intracellular polyamines by enhanced AO activity plays a critical role in PCD induced by UVB irradiation, adding a functional meaning to the results obtained with isolated mitochondria. Also intracellular accumulation of polyamines during pathophysiological conditions might increase the oxidation products generated by AO (mainly H_2O_2), possibly exceeding the scavenging ability of cells, and thus inducing PCD [3–8]. Keeping in mind that cytochrome *c* release and caspase activation are common to several apoptotic pathways triggered by unrelated stimuli [11], it can be speculated that these findings might have a broader validity and that AO activity might be a potential target of new therapies of apoptosis-related human pathologies.

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