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Participation of the mitochondrial permeability transition pore in nitric oxide-induced plant cell death

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Abstract In the present study, we investigated the involvement of the mitochondrial permeability transition pore (PTP) in nitric oxide (NO)-induced plant cell death. NO donors such as sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine inhibited growth and caused death in suspension-cultured cells of *Citrus sinensis*. Cells treated with SNP showed chromatin condensation and fragmentation, characteristic of apoptosis. SNP caused loss of the mitochondrial membrane electrical potential, which was prevented by cyclosporin A (CsA), a specific inhibitor of PTP formation. CsA also prevented the nuclear apoptosis and subsequent *Citrus* cell death induced by NO. These findings indicate that mitochondrial PTP formation is involved in the signaling pathway by which NO induces apoptosis in cultured *Citrus* cells.

Key words: Nitric oxide; Programmed cell death; Mitochondrial permeability transition pore; Apoptosis; *Citrus* cell

1. Introduction

Apoptosis, or programmed cell death (PCD), is the major physiological mechanism for selective cell elimination during development or following damage in multicellular organisms [1]. Characteristic morphological changes associated with PCD include cell shrinkage, chromatin condensation, nuclear DNA fragmentation and membrane blebbing [2]. Numerous death induction pathways elicited by different stimuli can trigger these morphological changes in cells undergoing the apoptotic program [3].

In mammalian cells, a variety of pro-apoptotic signals cause dissipation of the inner membrane electrical potential ($\Delta\Psi_m$) which favors the formation and opening of a permeability transition pore (PTP) through which apoptotic mitochondrial proteins are released into the cytosol [4–6].

Proteins released from mitochondria during apoptosis include cytochrome *c* and the apoptosis-inducing factor (AIF). AIF moves directly to the nucleus where it produces chromatin condensation and nuclear fragmentation. Cytochrome *c* activates a caspase signaling cascade that selectively cleaves vital substrates in the cell, including the nuclease responsible for DNA fragmentation [3,5].

Although the full structure of the PTP has not yet been elucidated, this pore is a polyprotein complex that includes inner membrane proteins such as the adenine nucleotide translocator, outer membrane proteins such as the voltage-dependent anion channel and cyclophilin D from the matrix [5]. The opening of this non-selective channel is regulated by the mitochondrial redox potential [7], as well as by endogenous effectors, including members of the *bcl-2* gene family [4].

Nitric oxide (NO), a potent radical oxidant, causes apoptosis in a variety of animal cells by triggering mitochondrial PTP formation [8]. Direct modulation of PTP opening [9] and the inhibitory effect of NO and its derivatives on mitochondrial respiration [10] are mechanisms implicated in the effect of NO on mitochondrial membrane permeability.

NO is also an important signaling molecule in plants. A role for NO in plant growth and development [11] and in defense responses against pathogens [12,13] has been suggested. NO production by the host activates the transcription of defense genes and triggers PCD to prevent the spread of the pathogen from the site of infection, in a process known as the hypersensitive response [14]. The PCD observed in the hypersensitive response in several plant–pathogen interactions exhibits similarities to that seen in animal apoptosis, including DNA fragmentation [15,16] and caspase-like proteolytic activity [17]. However, the involvement of the mitochondrial PTP in PCD in plants has not yet been addressed [18]. In this study, we provide evidence for participation of the mitochondrial PTP in NO-induced plant cell death.

2. Materials and methods

2.1. Cell cultures and treatment with NO donors

Suspension cultures of *Citrus sinensis* Obs. cv. Pera derived from nucellar calluses were grown in liquid medium (LM) as described [19]. The cultures were incubated with rotation (150 rpm) at 26°C in the dark, on a 14 day growth cycle. After 12 days in culture, the cells were harvested by suction filtration, weighed, the amount of cells adjusted to 0.7 g per 50 ml of LM. All procedures were done under aseptic conditions. The reagents used in the experiments were sterilized by filtration through a Millipore filter (0.22 μm). *S*-Nitrosoglutathione (GSNO) and nitrosocysteine (CYSNO) were synthesized as previously

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Abbreviations: CMXRos, chloromethyl X-rosamine; CsA, cyclosporin A; CYSNO, nitrosocysteine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; GSNO, *S*-nitrosoglutathione; $\Delta\Psi_m$, membrane electrical potential; PBS, phosphate-buffered saline; PCD, programmed cell death; PTP, permeability transition pore; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside

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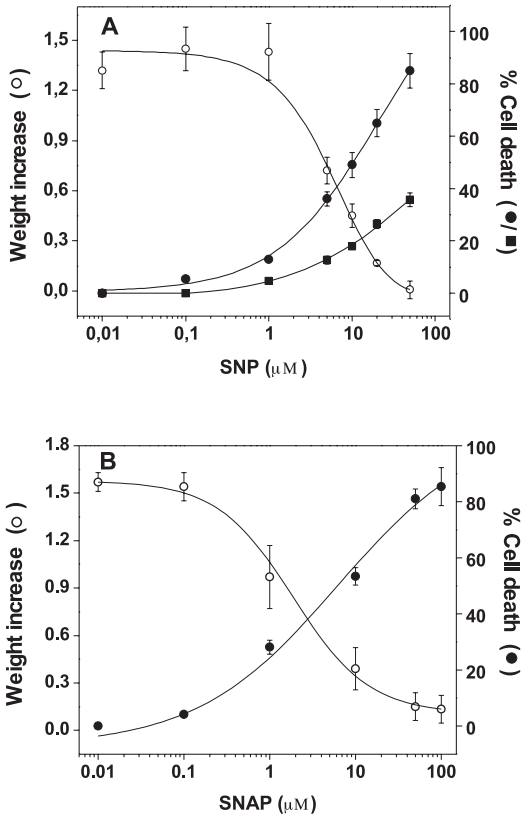

Fig. 1. Inhibition of *Citrus* cell proliferation by NO donors correlates with cell death. Suspension cultures (0.7 g/50 ml of liquid medium) were treated with the indicated concentrations of (A) SNP or (B) SNAP. After 10 days of treatment, cell death was estimated by Evans blue incorporation (closed circles), and cell proliferation was determined by measuring the fresh weight increase in the suspension cultures (open circles). In A, cell death was also estimated after 3 days of treatment with SNP (closed squares). The points are the mean values of two independent experiments done in duplicate; bars indicate S.D.

described [20]. NO donor solutions were prepared just before the experiments. Cellular growth was determined by the difference in the fresh weight of the suspensions after each treatment.

2.2. Assessment of cell death

Cell death was evaluated as described elsewhere [21]. Briefly, *Citrus* cells in suspension culture were filtered under vacuum and incubated for 15 min with 0.05% Evans blue in LM (90 mg or 100 mg of cells/ml). Unbound dye was removed by washing the cells six times in LM with centrifugation ($13\,000 \times g$, 2 min). Dye bound to dead cells was solubilized in 50% methanol with 1% SDS for 30 min at 50°C and quantified by the absorbance at 600 nm.

2.3. Nuclear labeling

Hoechst-33342 (Molecular Probes, Eugene, OR, USA) was used to detect chromatin condensation and DNA fragmentation. After different times of treatment, as specified in the figure legends, the cells were incubated with 1% potassium permanganate for 15 min and then with 2.5 μM Hoechst-33342 for 30 min. After nuclear DNA staining, the cells were washed three times with phosphate-buffered saline (PBS), pH 7.4, and viewed as described below.

2.4. Mitochondrial labeling

Mitotracker Red chloromethyl X-rosamine (CMXRos, Molecular Probes), a cell-permeant, mitochondria-specific fluorescent dye, was used to visualize the mitochondria. After the treatments specified in the figure legends, cells were washed and suspended in PBS, pH 7.4, and then incubated with CMXRos at a final concentration of 500 μM for 30 min at 25°C with shaking. After mitochondrial staining, the cells were washed in PBS (10 times, 5 min each), fixed with 4% paraformaldehyde in PBS for 30 min, and then washed in PBS before viewing as described below.

2.5. Confocal microscopy

Citrus cells stained with Hoechst-33342 or CMXRos were mounted in PBS containing 87% glycerol on slides covered with a coverslip and examined under a dual-channel laser confocal system (MRC 1024UV, Bio-Rad, Hercules, CA, USA) coupled to an Axiovert 100 inverted microscope (Zeiss) and equipped with Ar-Kr and UV lasers. The 568 nm line of the Ar-Kr laser was used for fluorescent excitation of Mitotracker Red CMXRos and the 350 nm line of the UV laser was used for Hoechst excitation. Manufacturer-supplied software (OS/2) running on a Pentium 150 MHz computer (Compaq) was used to control image acquisition and processing. To obtain quantitative data on the CMXRos fluorescence signal, the pixel values were measured in lines drawn across individual cells and expressed as arbitrary fluorescence units.

3. Results

3.1. NO donors inhibit growth and induce death of suspension-cultured Citrus cells

NO donors, such as sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), GSNO and CYSNO, dose-dependently inhibited the growth of suspension-cultured cells of *C. sinensis*. Growth inhibition was estimated by determining the fresh weight of the cultures after 10 days of exposure to increasing concentrations of NO donors. SNP ($IC_{50} = 5.0$ μM) and SNAP ($IC_{50} = 4.7$ μM) were very effective in inhibiting growth (Fig. 1), whereas GSNO ($IC_{50} = 565$ μM) and CYSNO ($IC_{50} = 870$ μM) inhibited growth only at much higher concentrations. The release of NO from GSNO and CYSNO is light-dependent [22], which may explain the very low effectiveness of these compounds on *Citrus* cell growth since the cells were cultivated in the dark.

Cell viability was used to determine whether the antiproliferative effect of NO donors in *Citrus* suspension cultures was due to growth arrest or cell death. As shown in Fig. 1, there was a clear correlation between cell death and the inhibition of growth by SNP and SNAP. Fig. 1A also shows that the percentage of cell death was much lower after 3 days compared to after 10 days of exposure to any of the SNP concentrations tested. Thus, NO donors caused a gradual decrease in *Citrus* cell survival.

3.2. Cell death induced by NO donors results in nuclear DNA fragmentation

To establish the type of cell death induced by NO donors, *Citrus* cells exposed to 10 μM SNP were fixed, stained with Hoechst-33342 and visualized by fluorescence microscopy. As shown in Fig. 2, control cells had a normal nuclear morphology (Fig. 2A), while in SNP-treated cells the nuclei were smaller and showed chromatin condensation and DNA fragmentation (Fig. 2B). Similar nuclear apoptotic features were also observed with 10 μM SNAP (Fig. 2C) and 500 μM GSNO (result not shown). Chromatin condensation and fragmentation prior to plasma membrane damage is a known characteristic of apoptosis and the time course of pycnotic nucleus formation after SNP treatment (Fig. 2E) preceded *Citrus* cell death, as shown by vital staining (Fig. 1A).

Nuclear pycnosis was prevented when the suspension cultures were treated with cyclosporin A (CsA) 1 h prior to exposure to the NO donors (Fig. 2D,E). Since CsA inhibits the opening of the mitochondrial PTP [7,23] this finding sug-

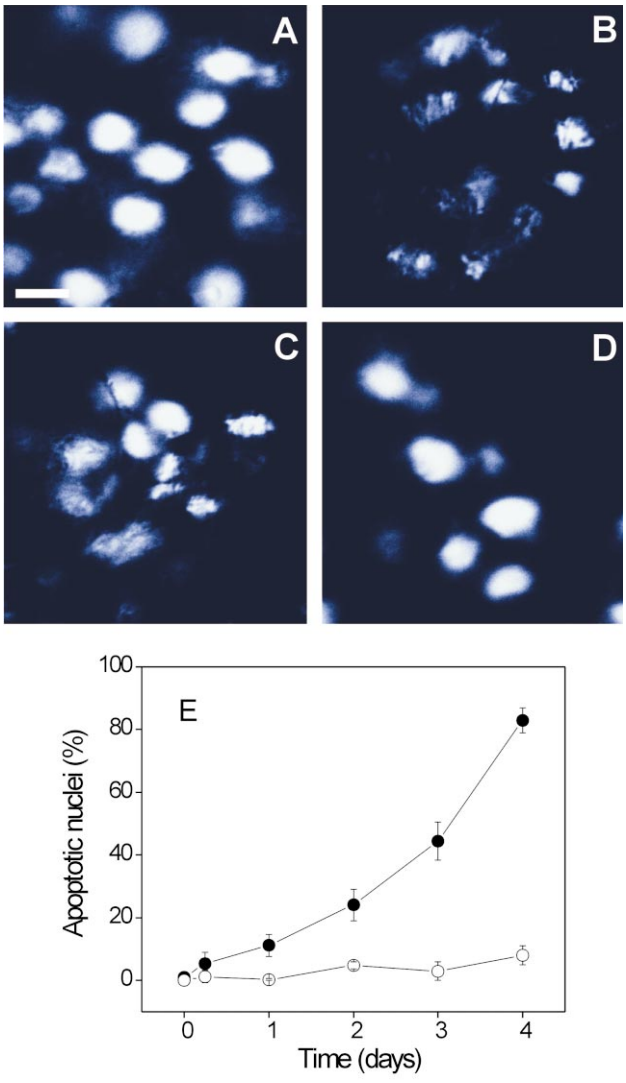

Fig. 2. NO donors induce nuclear apoptosis of *Citrus* cells that is prevented by CsA. Morphological alterations in chromatin were evaluated by Hoechst-33342 staining. A: Control. B: SNP 10 μM. C: SNAP 10 μM. D: CsA 1 μM for 1 h and then SNP 10 μM in 3 day cultures. Bar = 10 μm. E: Time course of nuclear fragmentation in cells treated with 20 μM SNP (closed circles) or 5 μM CsA and 20 μM SNP (open circles); apoptotic nuclei were counted on two slides with five fields per slide and each field containing ~100 cells.

gested that the mitochondrial dysfunction would be upstream in the NO signaling pathway leading to *Citrus* cell death.

3.3. *Mitochondrial dysfunction in Citrus cells exposed to NO donors*

To examine the participation of mitochondria in the NO-induced death of cultured *Citrus* cells, we evaluated the effect of NO donors on mitochondrial integrity. After 2 days of exposure to 10 μM SNP, the cells were incubated with the fluorescent probe CMXRos, fixed and analyzed using confocal microscopy to visualize the mitochondria. Mitochondrial labeling by CMXRos was markedly decreased in cultured cells exposed to SNP (Fig. 3B) compared to the controls (Fig. 3A), but was restored when CsA was present before SNP (Fig. 3C). The mitochondria of some cells treated with SNP showed spots of intense fluorescence (Fig. 3D) that probably represented major morphological alterations in this organelle. A similar effect has been observed in various animal cell lines undergoing apoptosis caused by free radicals [24,25].

Labeling of mitochondria by CMXRos was abolished when control cells were treated with the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) for 1 h (Fig. 3E). On the other hand, mitochondria of control cells treated for 24 h with the calcium ionophore A23187 (Fig. 3F) showed the same aspect as those exposed to SNP (Fig. 3D). These results indicated that the sudden collapse of the mitochondrial membrane electrical potential ($\Delta\Psi_m$), which leads to PTP opening, was not enough to induce the structural alterations in *Citrus* cell mitochondria seen after 2 days of exposure to SNP. However, such alterations could be mimicked by severe dysregulation of intracellular Ca^{2+} homeostasis, and Ca^{2+} accumulation by mitochondria triggers PTP opening [4].

Fig. 4 shows that the frequency distribution of the mean CMXRos signal shifted to lower values of fluorescence intensity in cells treated with 20 μM SNP for 2 days (Fig. 4B) compared to the control (Fig. 4A). In the absence of CsA, the loss of $\Delta\Psi_m$ was detected after 8 h of SNP treatment and increased gradually during the following 48 h (Fig. 4C). After

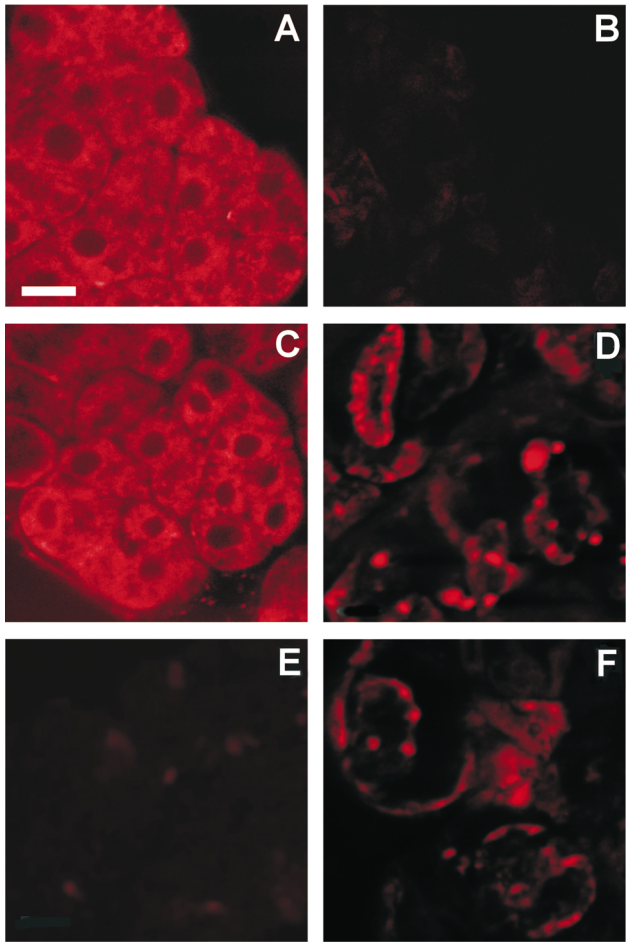

Fig. 3. CsA prevents the mitochondrial $\Delta\Psi_m$ loss induced by SNP. *Citrus* cells were stained with the potential-sensitive dye CMXRos after 2 days in culture. A: Control. B: SNP 10 μM. C: CsA 1 μM for 1 h and then SNP 10 μM. D: SNP 20 μM. E: Control cells treated with 10 μM of the protonophore FCCP for 1 h. F: Control cells treated with 10 μM of the Ca^{2+} ionophore A23187 for 24 h. Bar = 10 μm.

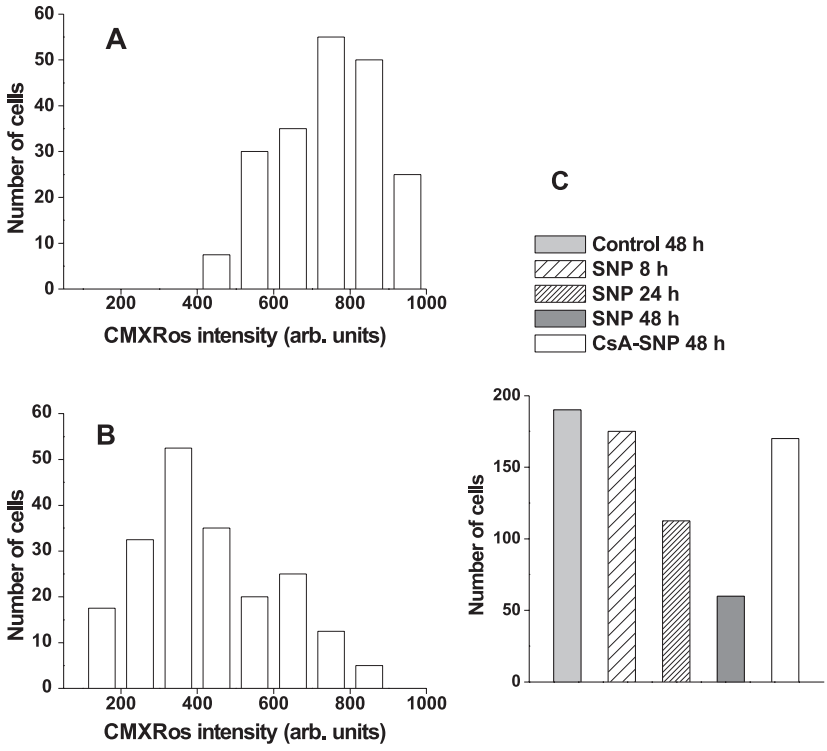

Fig. 4. Quantitative changes in the $\Delta\Psi_m$ of *Citrus* cells estimated by the relative CMXRos fluorescence intensity. The histograms in A and B show the frequency distribution of the mean CMXRos signal from cells grown in the absence (A) or presence (B) of 20 μM SNP for 2 days. C: Number of cells with a CMXRos fluorescence intensity in the range of 500–1000 arbitrary units (from a total of 200 cells counted per treatment).

3 days of treatment with SNP, the CMXRos signal, including the spots of fluorescence observed in some cells in the first 2 days of NO exposure, was completely abolished. At this time, as shown in Fig. 2E, less than 50% of the nuclei were fragmented. Thus, mitochondrial $\Delta\Psi_m$ loss preceded nuclear apoptosis in *Citrus* cells exposed to NO.

3.4. Cyclosporin A prevents growth inhibition and cell death induced by NO

Fig. 5A shows that the dose-dependent inhibitory effect of SNP on *Citrus* cell growth, evaluated after 10 days of exposure to the NO donor, was partially prevented by CsA. The inability of CsA to restore growth to control (pre-SNP) levels may reflect a toxic effect of long exposure to the drug since CsA per se showed a dose-dependent inhibitory effect on *Citrus* cell growth by this time. Assessment of cell death under these conditions showed that survival of *Citrus* cells was not affected until after 7 days of exposure to CsA. In addition, death induced in these cells by NO was prevented in the presence of CsA (Fig. 5B). These results indicated that, although CsA had some antiproliferative effect on *Citrus* cell growth, it was able to prevent the cell death induced by SNP.

4. Discussion

The multifunctional role of NO in mammals has stimulated an interest in the actions of this radical in plants. Although the plant NO synthase (NOS) enzyme has not yet been cloned, the cross-immunoreactivity of plant cells with NOS antibodies indicates that a homologous enzyme may exist in the plant kingdom [11]. NO has also been implicated in plant defense mechanisms against pathogens [12–14], with NO-induced PCD in infected cells limiting their spread. The major components of the signaling pathway by which NO triggers cell death in plant cells are poorly understood.

Here, we have shown that NO donor compounds inhibit the proliferation and reduce the survival of suspension-cultured cells of *C. sinensis* by a mechanism consistent with a mitochondrial-dependent apoptotic process. This conclusion was based primarily on the observation that the inhibitory effects of NO donors on *Citrus* cell growth in suspension cultures was preceded by functional and morphological alterations similar to those seen in the mitochondria of various animal cell lines undergoing mitochondrial apoptosis [25–27]. Subsequently, *Citrus* cells treated with NO donors presented chromatin condensation and fragmentation followed by a loss of viability.

CsA prevented NO-induced functional alterations in mitochondria (Figs. 3 and 4), as well as nuclear degradation of *Citrus* cells (Fig. 2). In agreement with these protective effects on mitochondrial and nuclear integrity, CsA also substantially delayed the antiproliferative action and the cell death induced by NO (Fig. 5). The ability of CsA to prevent NO-induced apoptosis in some animal cell types has been attributed to its inhibitory activity on mitochondrial PTP formation [8]. Thus, mitochondrial PTP formation may be involved in the signaling pathway of NO-induced *Citrus* cell death.

NO is not a universal inducer of apoptosis and, in some circumstances, can act as an anti-apoptotic agent [28]. This pleiotropic nature of NO may be explained by differences in the components of the apoptotic machinery or by the ability of the cells to degrade or inactivate NO. These factors may partly explain the variations in the susceptibilities of cell types to NO toxicity [29]. NO is pro-apoptotic in *Citrus* cells, and

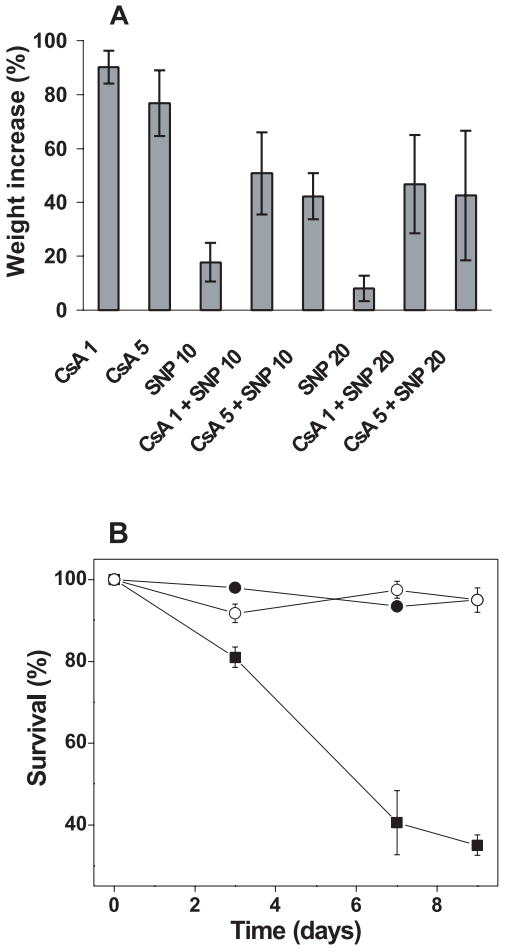

Fig. 5. SNP inhibition of *Citrus* cell proliferation and the induction of cell death are prevented by cyclosporin A. A: Proliferation of suspension cultures (0.35 g/25 ml of liquid medium) after 10 days of treatment with CsA, SNP or CsA for 1 h and then SNP. The concentrations used were: 10 μM or 20 μM for SNP and 1 μM or 5 μM for CsA. B: Time course of cell survival in the absence (open circles) or presence (closed squares) of 20 μM SNP or 5 μM CsA and 20 μM SNP (closed circles). The data shown are the mean values of two independent experiments done in duplicate; bars indicate S.D.

PTP formation in the mitochondrial membrane is one of the molecular targets modified in the presence of NO.

NO donors such as SNP and SNAP have a very short half-life when added to culture media. The observation that the collapse of the mitochondrial $\Delta\Psi_m$ started only after 8 h of treatment with SNP suggests that mitochondria, instead of being the immediate target of NO, would participate in the amplification of the stress signals triggered by this nitrogen radical in these cells. The components of the signaling cascade triggered by NO donors in *Citrus* cells leading to mitochondrial PTP opening have not yet been identified.

Prototypic regulators of animal cell death, such as Bcl-2 and its homologues, exert their anti- or pro-apoptotic actions by controlling the opening of the mitochondrial PTP. Bcl-2 prevents PTP opening and consequent cytochrome *c* release, whereas Bax counters the actions of Bcl-2, probably by promoting cytochrome *c* release [5,27]. Plants apparently do not have Bcl-2-like proteins. The absence of such a regulatory mechanism for controlling apoptosis could account for the high sensitivity of *Citrus* cells to NO, and could also explain why NOS activity in plant cells may be sufficient to prevent the spreading of pathogens from the site of infection.

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