Nitric oxide affects plant mitochondrial functionality in vivo

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Abstract In this report, we show that nitric oxide affects mitochondrial functionality in plant cells and reduces total cell respiration due to strong inhibition of the cytochrome pathway. The residual respiration depends on the alternative pathway and novel synthesis of alternative oxidase occurs. These modifications are associated with depolarisation of the mitochondrial membrane potential and release of cytochrome *c* from mitochondria, suggesting a conserved signalling pathway in plants and animals. This signal cascade is triggered at the mitochondrial level and induces about 20% of cell death. In order to achieve a higher level of cell death, the addition of H_2O_2 is necessary.

Key words: Nitric oxide; Mitochondrion; Alternative oxidase; Oxidative stress; Programmed cell death; *Daucus carota*

1. Introduction

The importance of nitric oxide (NO) as a signalling molecule in many biological processes is becoming increasingly evident. In mammalian systems, NO has been shown to have diverse roles in a number of different cell types [1]. In plants, it is at the heart of several physiological functions, ranging from development to defense mechanisms that are triggered during the hypersensitive response (HR) [2]. In mammals, NO is produced by nitric oxide synthase (NOS) [3] and the existence of a plant equivalent to NOS [4] suggests that the mechanism of action of NO is conserved in plants and animals. Recent studies in animal systems have demonstrated that NO can directly interact with mitochondria by inhibiting complex IV of the respiratory chain [5] and can also induce the release of various proteins, normally sequestered in the mitochondrial intermembrane space, such as cytochrome *c* and apoptosis-inducing factors [6]. In plants, NO appears to be a signalling molecule of vital importance for several cell responses [7], although the target and the mechanisms involved in these processes have yet to be understood.

NO is known to be involved in the hypersensitive reaction that is activated upon pathogen attack. An early and characteristic event of the HR is the production of reactive oxygen species [8]. Recently, it has been observed that H_2O_2 is not sufficient to trigger the HR, although it might act in conjunction with other factors to activate cell death [9]. Several experiments in plants and in cell culture have suggested that NO is one such factor. In fact, during the HR, NO increases the amount of cell death induced by H_2O_2, augmenting the effect of the oxidative burst and stimulating the expression of pathogenesis defense genes [10].

In this paper, we describe the effects of the NO-donor sodium nitroprusside (SNP) on the viability of carrot cells and, in particular, on mitochondrial functionality, ascribing to mitochondria an important role in NO-induced cell death process. Our results aid in interpreting the synergistic action of NO and H_2O_2 and imply the existence of a conserved signalling pathway between plants and animals.

2. Materials and methods

2.1. Cell cultures, treatments and viability

Carrot (*Daucus carota* cv. S. Valery) cells in suspension were cultivated in Gamborg's B5 medium as previously described [11]. Treatment with SNP, glucose 0.5 mM+glucose oxidase 0.1 U/ml (g/go) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Alexis, San Diego, CA, USA) were performed on 3-day-old cells. Cell death was determined by spectrophotometric measurements of the uptake of Evan's blue as described [12]. Unless otherwise indicated, all chemicals were purchased from Sigma.

2.2. Microscopy

Protoplasts were obtained as described in [11] and stained with 2 μg/ml 4,6-diamino-2-phenylindole (DAPI; Sigma-Aldrich). They were subsequently visualised using a Nikon Epi-fluorescence microscope with an excitation filter of 330–380 nm and barrier filter of 400 nm.

2.3. Measurement of oxygen consumption

1.3 ml of cell suspension (approximately 100 mg fresh weight) were analysed for O_2 consumption in a glass chamber, maintained at 25°C, equipped with a Clark-type oxygen electrode (Rank Brothers, Bottisham, UK) connected to a chart recorder. The oxygen electrode was calibrated with air-saturated medium kept at 25°C, assuming an oxygen concentration of 240 μM. Antimycin was used as an inhibitor of the complex III of the respiratory chain at the final concentration of 5 μM. Salicylhydroxamic acid (Sham) was used to inhibit the alternative oxidase (AltOx) at a final concentration of 1 mM [13].

2.4. Protein extraction and analysis

Proteins were extracted as previously described [14]. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay; Bio-Rad, Germany). Mitochondrial and cytoplasmic pro-

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Abbreviations: AltOx, alternative oxidase; COX, cytochrome oxidase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAPI, 4,6-diamino-2-phenylindole; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; MMP, mitochondrial membrane potential; NOS, nitric oxide synthase; Sham, salicylhydroxamic acid; SNP, sodium nitroprusside; TMRM, tetramethylrhodamine

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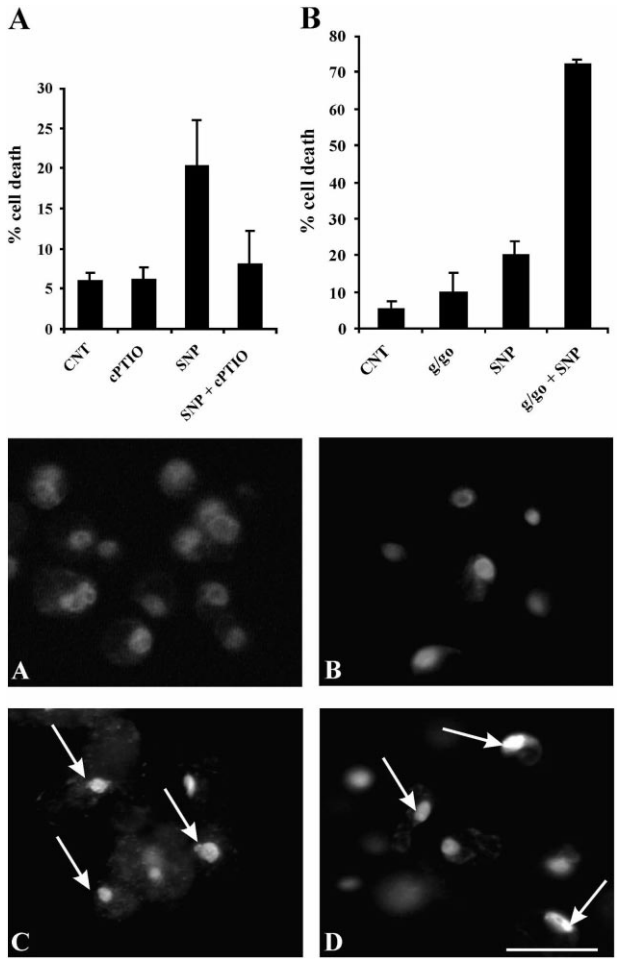

Fig. 1. Lethal effect of SNP. Top panel, viability of cells: A: Treated for 24 h with 1 mM SNP and/or 1 mM cPTIO; B: treated for 48 h with g/go and/or 1 mM SNP. Data are reported as mean values ± S.D. Lower panel, chromatin condensation in carrot cells: A: untreated, B: treated with g/go, C: treated with SNP or D: treated with SNP+g/go stained with DAPI. Arrows indicate representative granular nuclei. Scale bar = 40 μm.

teins were separated by 15% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Sartorius, Italy), and analysed with antibodies raised against human cytochrome *c* (Santa Cruz, Germany). Immunodetection of the AltOx was carried out using monoclonal antibodies, kindly provided by Dr. T.E. Elthon, University of Lincoln-Nebraska [15]. Uniform transfer of proteins was controlled by staining with Ponceau S. Densitometric analysis of the blots were performed with a digital imaging analysis system (Chemi Doc, Bio-Rad).

2.5. Fluorescence microscopy

Aliquots of cells were incubated at 25°C for 15 min in B5 culture medium containing 500 nM tetramethylrhodamine (TMRM), washed three times with B5 medium, and layered on coverslips which had been pre-treated for adhesion for 10 min with poly-L-lysine. Cell fluorescence images acquisition and data analysis were performed as previously described [16].

3. Results

3.1. Effect of nitrosative stress on cell viability

In initial experiments, we studied the viability of cells treated with SNP as a chemical donor of NO. Treatment of suspension cultures of carrot cells with SNP (1 mM) induced a decrease in cell viability, corresponding to about 20%, after 24 h. In order to verify that NO was the causative agent, these experiments were carried out in the presence of a NO scavenger, cPTIO. As shown in Fig. 1A (top panel), cPTIO prevents the killing effect of NO, thus demonstrating that NO, rather than the entire donor molecule, is responsible for cell lethality. By itself, cPTIO had no effect on cell viability. In Fig. 1B (top panel), the synergistic effect of NO and H_2O_2 in inducing cell death is shown and it is evident that 0.1 U/ml g/go causes a reduction in cell viability of about 10% after 24 h. The combined treatment with SNP and g/go results in a massive cell death (72%) after 24 h. In order to evaluate the 'nature' of this death we stained the cells with DAPI to check for the localised condensation of chromatin, characteristic of PCD [17]. This feature was evident in cells treated with SNP or SNP+g/go (Fig. 1, lower panel).

3.2. Effect of NO on the respiration of carrot cells

In the experiments detailed in Fig. 2A, carrot cells were

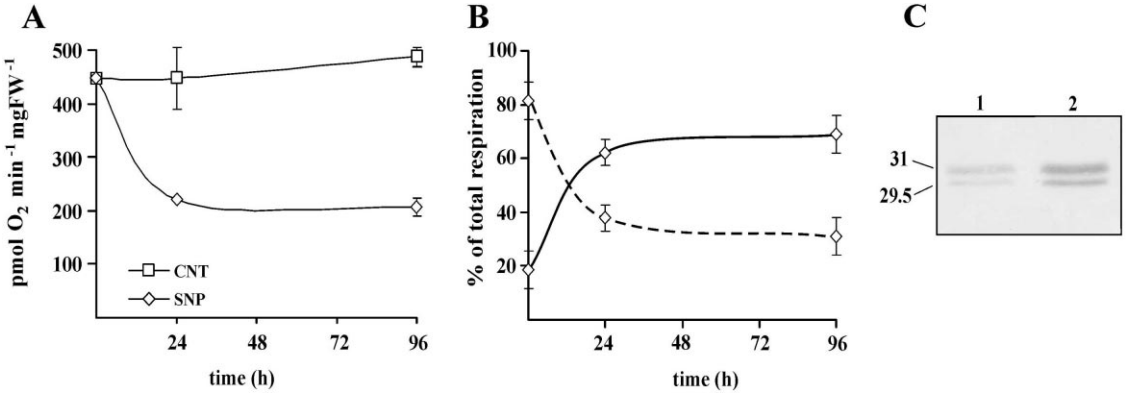

Fig. 2. Effect of nitrosative stress on mitochondrial respiration. A: Inhibitory effect of 1 mM SNP on total respiration compared to control cells. B: Respiration of SNP-treated carrot cells in the presence of Sham (broken line) or antimycin (continuous line). The alternative and cytochrome components to total respiration of control untreated coincide with the value at time $t = 0$ of the graph. C: AltOx immunodetection in mitochondrial extracts of untreated cells (1) and cells treated with 1 mM SNP for 24 h (2). 20 μg of mitochondrial proteins were loaded on each lane. Data are presented in A and B as mean values ± S.D.

incubated with 1 mM SNP and the respiratory activity was measured at different times using a Clark-type O_2 electrode. It can be seen that treatment with 1 mM SNP reduced total cell respiration to approximately 50% within 24 h. Total respiration depends on the cytochrome and alternative pathways. The latter branches from the cytochrome pathway at the ubiquinone pool and it is only linked to proton translocation at the level of complex I (NADH dehydrogenase). This pathway is characteristic of plants, some fungi and protists [13].

The decrease in respiration observed in Fig. 2A may depend on inhibition of COX or the AltOx, or both. We then determined the contribution of SNP-dependent inhibition of the two pathways to total respiration by adding 1 mM Sham to inhibit the alternative pathway, or 5 μM antimycin to inhibit the complex III of the respiratory chain. Fig. 2B shows the decreasing contribution of the cytochrome pathway to total respiration, while the AltOx pathway contribution seems to be more important after SNP treatment. We next ascertained whether the increased contribution of the AltOx was due to a diversion of electrons from the inhibited cytochrome pathway to the alternative pathway or if the induction kinetic of the latter pathway could be attributed to an increased expression of the AltOx complex. In order to address this point, we performed Western blot analysis using anti-AltOx antibodies. The results, shown in Fig. 2C, demonstrate that the amount of antibody reactive protein increases after addition of SNP. The two stained bands show apparent molecular masses of 29.5 and 31 kDa, which correspond to the proteins of the AltOx cluster previously identified in other plant species [18]. The inhibition of the cytochrome pathway is consistent with what is seen in animal systems, in that NO binds to cytochrome *c* oxidase (COX, the terminal enzyme of the mitochondrial respiratory chain) which controls cellular functions via reversible inhibition of respiration [19]. The NO-specificity of this effect has been verified with cPTIO. The results of these experiments are presented in Fig. 3A, where O_2 consumption is reported as percent of total respiration with and without Sham.

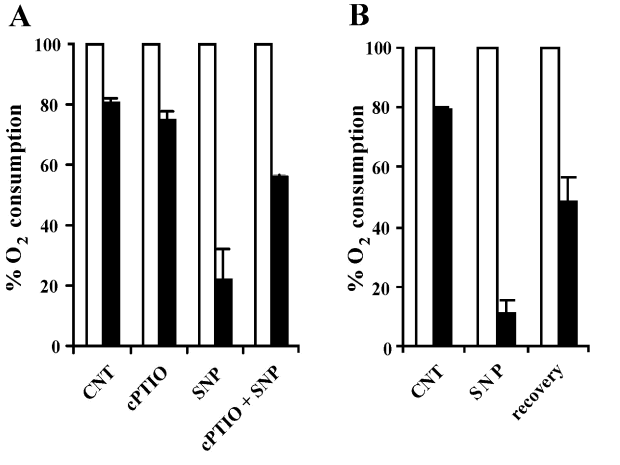

Fig. 3. Specific and reversible effects of NO on cell respiration. A: Effect of 24 h treatment with SNP and cPTIO on respiration of carrot cells. B: Recovery process (4 days after 72 h of SNP treatment) of respiration upon NO-donor removal. The values, reported as percent of total respiration of control and treated cells, refer to cells without (open columns) or with (closed columns) Sham. Data are reported as mean values ± S.D.

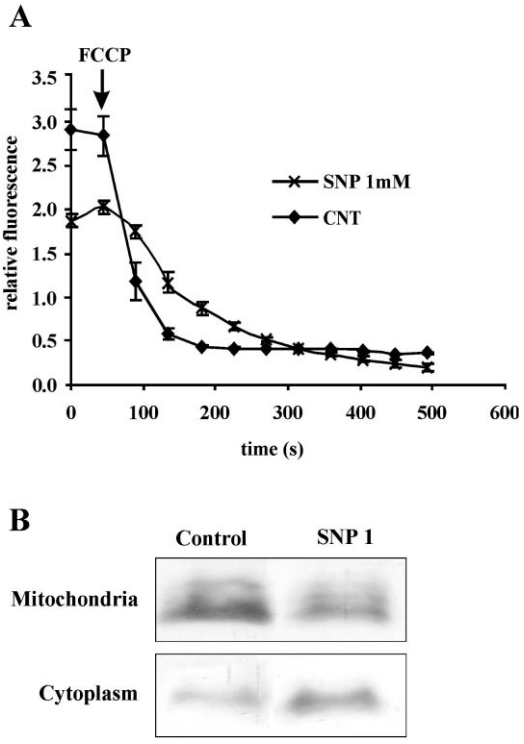

Fig. 4. Effect of nitrosative stress on mitochondrial membrane. A: Variation of TMRM fluorescence of cells treated with SNP (1 mM) for 1 h. The arrow indicates the time of addition of 500 nM FCCP. Scale bar = 50 μm. Data (mean of three independent experiments) are reported as mean values ± S.D. B: Cytochrome *c* mobilisation in cells after 5 h of treatment with SNP. Cytochrome *c* immunodetection in mitochondrial and cytosolic protein extracts (80 μg) of SNP-treated and untreated control cells.

In order to determine if the inhibition of the cytochrome component of cell respiration is reversible, we treated cells with 1 mM SNP for 72 h, and then replaced the medium with a pre-conditioned medium (conditioned by cells of the same age) without SNP. As seen in Fig. 3B, after 4 days of recovery in the pre-conditioned medium, the effects of NO were partially reversed.

3.3. *Effect of nitrosative stress on mitochondrial membrane potential (MMP)*

We next set up an in vivo method to measure the membrane electrical potential of plant mitochondria ($\Delta\Psi_m$) which, in animals, is known to be affected by NO [20]. The experimental approach is based on the measurement of the extent of the membrane potential-driven import of the fluorescent cationic dye TMRM [16]. When incubated with TMRM, carrot cells display a clear mitochondrial fluorescence pattern (not shown). The accumulation of the dye involves only the mitochondria and depends on their energisation state. This is demonstrated by adding the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, 500 nM) which, by collapsing the electrical component of the membrane potential ($\Delta\Psi$), causes a decrease in the fluorescence signal to a minimum level corresponding to completely de-energised mitochondria (not shown). The fluorescence variation before and after adding FCCP is thus the measure of the percentage of energisation. In Fig. 4A, the TMRM fluorescence of cells treated for 1 h with SNP is compared with untreated cells.

SNP treatment induces a reduction of the programmed cell death resulting from the fluorescence signal, demonstrating that NO causes a decrease in MMP.

3.4. Cytochrome c release from mitochondria

The above data suggest an important role of mitochondria in NO-induced cell responses and it was thus of interest to determine if the involvement of mitochondria also implies the release of apoptotic factors such as cytochrome *c*. Fig. 4B shows Western blot analysis of mitochondrial and cytosolic proteins extracted from SNP-treated cells using anti-cytochrome *c* antibodies. Densitometric analysis revealed a reduction of mitochondrial cytochrome *c* level of about 21% upon treatment with 1 mM SNP. This effect is already apparent 5 h after treatment.

4. Discussion

In this report, we demonstrate that NO has specific effects at the mitochondrial level in carrot cell suspensions. From the available information about animal systems, mitochondria are one of the earliest targets of NO which reversibly, and competitively with O_2, inhibits their COX, and the same was found in isolated plant mitochondria [21]. Our aim was to understand the action of NO on cell viability and how this is mediated by mitochondria: to this extent, we decided to use an in vivo system of suspension cell culture.

Our results demonstrate that NO inhibits total respiration and, in particular, the cytochrome pathway. The high sensitivity of SNP-treated cells to Sham, with respect to untreated cells, suggests that the major contribution to the residual respiration (upon NO treatment) comes from the alternative pathway (Fig. 2B). Western blot analysis showed that the induction kinetics of the alternative pathway was associated with an increase of AltOx protein expression (Fig. 2C) and was not due only to diversion of electrons to AltOx. It is known that when the cytochrome pathway is inhibited, electron transport is shunted into the alternative pathway resulting in a nearly normal rate of respiratory O_2 uptake [22]. The same authors also reported that cytochrome pathway inhibitors such as antimycin A and cyanide induce AltOx expression. For the first time, we have shown that NO induces an increase in AltOx expression in plant cell cultures.

From the above results, a possible action mechanism of NO in plant cells can be inferred. By inhibiting COX-dependent respiration, SNP treatment decreases the capacity of the cytochrome pathway and enhances the contribution of the alternative pathway to total respiration by inducing the expression of the AltOx. These alterations are associated with the NO-dependent depolarisation of MMP, and release of cytochrome *c* from mitochondria.

It is already recognised that the decrease of MMP is an important mechanism associated with apoptosis in animal systems [23] and that cytochrome *c* release serves as a signal in several forms of programmed cell death in both animals and plants [4,24]. In our system, NO triggers a set of alterations typically associated with PCD as shown by nuclear condensation presented in Fig. 1 (lower panel). In animal systems, the consequence would be a very high level of cell death. However, in our plant system NO reduces cell viability by only 20%. We suggest that the distinctive features of the plant respiratory chain (i.e. the presence of the AltOx) account for the apparent resistance of plant cells. In fact, AltOx presence actually helps to prevent an overreduction of the electron-transport component of the respiratory chain [25] by reducing stress and allowing cells to survive until NO is removed.

As reported in the literature [9], in conjunction with an oxidative burst, NO induces a high level of cell death. In our experiments, compared with cell death induced by SNP alone, treatment with SNP and the H_2O_2 generating system g/go cause a four-fold increase in cell death. The limited lethal effect of H_2O_2 when given alone is probably due to the efficient detoxifying systems present in the cells that act as scavengers. In the present report, we demonstrate that NO alters mitochondrial functionality, thus affecting one of the scavenging systems of the cell [25]. In this light, the combined action of NO and H_2O_2 can be seen as the consequence of the altered redox balance of the cell.

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