Alterations in pyrimidine nucleotide metabolism as an early signal during the execution of programmed cell death in tobacco BY-2 cells

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Abstract
Changes in pyrimidine metabolism were investigated during programmed cell death (PCD) of tobacco BY-2 cells, induced by a simultaneous increase in the endogenous levels of nitric oxide (NO) and hydrogen peroxide. The de novo synthesis of pyrimidine nucleotides was estimated by following the metabolic fate of the 14C-labelled orotic acid, whereas the rates of salvage and degradation pathways were studied by measuring the respective incorporation of 14C-labelled uridine and uracil under different treatments. Nucleic acid metabolism was also examined using labelled thymidine as a marker. The results show that specific alterations in the balance of pyrimidine nucleotide synthesis, which include a decreased rate of salvage activity of uracil and uridine and increased salvage activity of thymidine, represent a metabolic switch that establishes proper cellular conditions for the induction of PCD. In particular, a reduction in the utilization of uracil for salvage products occurs very early during PCD, before the appearance of typical cytological features of the death programme, thus representing an early metabolic marker for PCD. These changes are strictly associated with PCD, since they do not occur if NO or hydrogen peroxide are increased individually, or if actinomycin, which inhibits the death programme, is added into the medium in the presence of NO and hydrogen peroxide. The possible roles of these fluctuations in pyrimidine metabolism on the cellular nucleotide pool are discussed in relation to the induction of cell death.

Key words: Programmed cell death, pyrimidines, tobacco BY-2.

Introduction
Programmed cell death (PCD) is a physiological process which regulates cell homeostasis through the elimination of unneeded cells, including those that have served temporary functions or those present in inappropriate positions. This deliberate elimination of cells appears to be indispensable for correct development in animals, as it contributes to cell specialization, tissue and organ determination, and tissue patterning. Mutations disrupting one or more events of PCD often result in morphological aberrations and premature death (White et al., 1994). By contrast with necrosis, execution of PCD relies on a fine network of genetic signals which lead to cellular suicide through the activation of precise autolytic processes (Wadewitz and Lockshin, 1988; Ellis et al., 1991; Beers and McDowell, 2001).

In plants, genetic programmes for cellular suicide, analogous to those observed in animals, can be activated as important components of the developmental programme or as defence mechanisms. During the hypersensitive response triggered by incompatible plant–pathogen interaction, for example, activation of PCD at the infection site limits the advance of the pathogen by restricting nutrient availability. Recent work has demonstrated that execution of PCD during pathogen attack is triggered by the interaction of signalling molecules, which include reactive oxygen such as hydrogen peroxide (H2O2), and nitric oxide (NO) (Delledonne et al., 2001). Production of H2O2, generated by an oxidative burst at the infection site, can have multiple effects, ranging from the transcriptional activation of defence genes, including glutathione-S-transferase and phenylalanine ammonia lyase (Lamb and Dixon, 1997; Lam et al., 1999), to structural modifications of the cell wall (Bolwell, 1999). This latter event is mediated by apoplastic peroxidases,
which participate in cross-linking structural cell wall components, thereby reinforcing the cell wall and containing the pathogen advance (Ros Barcelo, 1997).

Production of H$_2$O$_2$ at the infection site is necessary, but not sufficient, to trigger PCD. Another regulatory molecule which, in synergy with H$_2$O$_2$ but through independent regulatory mechanisms, participates in the activation of hypersensitive cell death, is NO (Noritake et al., 1996; Delledonne et al., 1998). In both soybean and tobacco BY-2 cells, activation of PCD requires a balance between H$_2$O$_2$ and NO, such that high levels of one molecule are ineffective in inducing hypersensitive cell death in the absence of the other (Delledonne et al., 2001; de Pinto et al., 2002).

The perception phase of the cell death signal is followed by the dismantling phase, which involves the degradation of many cellular components through the activation of autolytic mechanisms. Cytological hallmarks of the dismantling phase include the condensation, shrinkage, and fragmentation of cytoplasm, deterioration of membranes, and degradation of chromosomal DNA into small oligonucleosomal fragments. This latter event, a common feature of several types of PCD described so far, is the result of pronounced nuclease activity which is induced as a part of the cell death signal. In mouse FM3A cells, activation of nuclease activity followed by DNA fragmentation and cell death, can be experimentally induced by the exogenous application of 5-fluorodeoxyuridine, which alters the intracellular deoxyribonucleoside triphosphate pool (Yoshioka et al., 1987). This result leads to the speculation that an imbalance of nucleotide metabolism may represent an early key regulatory signal which initiates developmental programmed cell death.

In order to test this hypothesis, the relationship between NO and H$_2$O$_2$-mediated PCD and pyrimidine nucleotide metabolism was investigated in tobacco BY-2 cells. The activities of the de novo, salvage, and degradation pathways of pyrimidine metabolism were estimated by following the metabolic fate of the exogenously supplied $^{14}$C-labelled orotic acid, uracil, uridine, and thymidine, as well as by measuring the activity of major key enzymes. The results suggest that alterations in the contribution of de novo and salvage pathways to nucleotide biosynthesis caused by the interaction of NO and H$_2$O$_2$, can be part of a key regulatory signal that promotes PCD in plant cells.

Materials and methods

Plant material

The tobacco (Nicotiana tabacum L. cv. BY-2) cells were cultured in Murashige and Skoog (1962) medium supplemented with 370 mg KH$_2$PO$_4$, 1 mg l$^{-1}$ thiamine-HCl, 3% (w/v) sucrose, and 0.2 mg l$^{-1}$ (1 µM) 2,4-dichlorophenoxy-acetic acid, adjusted to pH 5.8. Manipulations of culture conditions and generation of NO and H$_2$O$_2$ were carried out as reported previously (de Pinto et al., 2002). Briefly, a stationary culture was diluted 2:100 (v/v) in fresh medium and agitated at 130 rpm in the dark at 22 $^\circ$C. At day 3, NO and H$_2$O$_2$ were generated by applications of 0.5 mM sodium nitroprusside (SNP) and glucose (0.4 mM) plus glucose oxidase (0.4 unit ml$^{-1}$) (GOG), respectively. Where indicated, actinomycin (1 µg ml$^{-1}$) was added to the cells at the same time as SNP and GOG supplements. Cells during the exponential phase of growth (3 d) were treated for 2, 4, and 8 h with SNP, GOG, or SNP+GOG, and incubated with radioactive pyrimidine precursors for the same length of time.

Cell growth parameters and microscopy

Determination of packed cell volume was carried out by centrifuging the cells at 260 g for 5 min and by calculating the ratio between cell volume and total suspension volume.

The mitotic index was determined as described in de Pinto et al. (1999). Briefly, cells were fixed for 30 min in Carnoy’s fixative, centrifuged at 260 g for 5 min, and washed three times in water. Cells were incubated with 18% HCl for 20 min, washed three times in water, and stained for 30 min in Schiff’s reagent (pH 2.2). For each treatment four slides were analysed with 600 cells scored for each slide.

Cell viability following treatments was estimated by using Trypan Blue, as reported previously (de Pinto et al., 1999). Cells (0.2 ml) were incubated for 10 min in 0.3 ml of 3% (w/v) sucrose and 0.5 ml of 4% trypan blue solution. The ratio between unstained (viable) cells and stained (dead cells) was used as an estimation of cell viability. For each treatment four slides were used and 600 cells per slide were scored.

Student’s t-test on nuclear morphology were carried out with DAPI stain, as reported by Callard et al. (1996). After fixation with 100 mM PIPEs, pH 6.8, 10 mM EGTA and 10 mM MgSO$_4$(PME buffer) containing formaldehyde (4% v/v), cells were washed in PME buffer and resuspended in PME buffer containing Triton X-100 (0.2% w/v) and 1 µg ml$^{-1}$ DAPI. Observations were carried out using a fluorescent microscope (Leica DMR HC) with an excitation filter of 340–380 nm and a barrier filter of 400 nm.

Production and visualization of NO was monitored as described by Privat et al. (1997) and Garces et al. (2001), whereas H$_2$O$_2$ production was measured as indicated by Bellicampi et al. (2000).

For cytological studies, control and treated cells were fixed in 2.5% glutaraldehyde and 1.6% p-formaldehyde buffered with 0.05 M phosphate buffer, pH 6.9, dehydrated with methyl cellosolve, followed by two changes of absolute ethanol, and then infiltrated and embedded in Historesin (Leica Canada, Toronto) (Yeung, 1999). Serial 3 µm sections were cut with glass knives on a Leica RM 2145 microscope. Sections were stained with DAPI stain and counterstained with 0.05% (w/v) toluidine blue O in benzene buffer, pH 4.4 (Yeung, 1999).

Metabolism of labelled pyrimidine precursors

The de novo and salvage synthesis and degradation of pyrimidine nucleotides were investigated by following the metabolic fate of radiolabelled pyrimidine intermediates. [2-$^{14}$C]orotic acid (specific activity, 1.85 MBq µmol$^{-1}$) was used as an intermediate of the de novo synthesis, whereas [2-$^{14}$C]uridine (specific activity 2.00 MBq µmol$^{-1}$) and [2-$^{14}$C]uracil (specific activity 1.85 MBq µmol$^{-1}$) were utilized as precursors of the salvage and degradation pathways, respectively (Fig. 1). DNA metabolism was also investigated by following the metabolic fate of exogenously supplied [2-$^{14}$C]thymidine (1.85 MBq µmol$^{-1}$). Administration of radiolabelled compounds was carried out according to a procedure described in a previous paper (Ashihara et al., 2000). Cells (approximately 150 mg) were incubated in the presence of 10 µl of labelled compounds (37 kBq) for 2, 4, and 8 h at 22°C. Extraction and analysis of labelled metabolites was performed as described by Ashihara et al. (2000). The samples were extracted with cold 6% perchloric acid (PCA). The supernatant represents the PCA-soluble fraction. The precipitate was then hydrolysed with 6% PCA at 100 °C for 20 min to give the nucleic acid fraction. The summation of radioactivity found in these two fractions plus labelled CO$_2$ released was designated as total uptake.
Preparation and assay of enzymes

For enzyme assays cells (approximately 500 mg) at different hours of incubation and with different treatments were utilized. Extraction and determination of specific activities of orotate phosphoribosyltransferase (OPRT), uracil phosphoribosyltransferase (UPRT), uridine kinase (URK), and nucleoside phosphotransferase measured with uridine (NTP (UR)), were performed as described by Ashihara et al. (2000). Activities of thymidine kinase (TRK) and nucleoside phosphotransferase measured with thymidine (NTP (TR)) were carried out as reported by Mullin and Fites (1978).

Statistical analysis

The Student’s t-test was used to determine statistical differences between values of control and treated cells.

Results

Effects of NO and \( \text{H}_2\text{O}_2 \) on tobacco BY-2 cells

The growth pattern of tobacco BY-2 cells, estimated by measurements of packed cell volume, had a sigmoidal curve with an exponential phase between days 3 and 5 and a subsequent stationary phase during the last days in culture. A peak of mitotic activity was observed at day 4, in conjunction with an increase in cell growth which started at day 2 (data not shown). These growth parameters are very similar to those reported in other studies (Nagata et al., 1992; Francis et al., 1995; de Pinto et al., 1999).

Pronounced cell death at the onset of the exponential phase (day 3) was induced by the combined applications of the NO donor SNP (0.4 mM) and the \( \text{H}_2\text{O}_2 \) generator glucose plus glucose oxidase (GOG; glucose 0.4 mM and glucose oxidase 0.5 units ml\(^{-1}\)), and actinomycin (ACT, 1 \( \mu \)g ml\(^{-1}\)) on cell viability of tobacco BY-2 cells. Values represent mean ± SE of at least three independent experiments. (B) Visualization of NO accumulated in tobacco BY-2 cells in the absence (control) or in the presence (SNP) of sodium nitroprusside (0.4 mM).
2002), were applied as they affected cell viability in a slow fashion (Fig. 2A). Cell death, which became visible after only 4 h in the presence of GOG+SNP, did not occur if SNP and GOG were supplied individually and was significantly reduced when treatments were performed in conjunction with actinomycin (Fig. 2A). At the concentrations used, the addition of the H₂O₂ generator GOG resulted in an accumulation of 5 μM H₂O₂ for more than 2 h, whereas SNP produced a steady-state accumulation of 2.5 μM NO (data not shown). Accumulation of NO in SNP-treated tobacco cells was also detected in intact cells (Fig. 2B).

Cytological studies were conducted using light and fluorescence microscopy on control and treated cells. Control cells and cells treated with either GOG or SNP had a normal appearance and were characterized by thin cell walls and large vacuoles. The nuclei of these cells stained uniformly with DAPI and had a large nucleolus surrounded by chromatin which had a smooth appearance (Fig. 3). Cells treated with GOG+SNP exhibited several morphological abnormalities, including shrinkage of the cytoplasm, condensation of chromatin which resulted in nuclei with granular appearance, and invaginations of the plasma membrane which became detached from the wall. These abnormalities were not visible if actinomycin was applied to these cells (Fig. 3).

Pyrimidine metabolism during PCD

Studies on de novo synthesis, salvage, and degradation of pyrimidine nucleotides in tobacco BY-2 cells were conducted by following the metabolic fate of ¹⁴C-labelled orotic acid, uridine, and uracil (Fig. 1). Nucleic acid metabolism was also estimated by monitoring the incorporation of labelled thymidine.

Total uptake of pyrimidine precursors

After 2 h, both control and treated cells were able to take up pyrimidine precursors. Differences between treatments were only observed for uridine, which showed higher uptake values in control cells (almost 100 nmol g⁻¹ fresh weight), compared to treated cells (SNP, GOG, and SNP+GOG) (Fig. 4). No statistical differences in uptake were observed for the other precursors.

Fig. 3. (A) Cellular morphology of tobacco BY-2 cells treated with SNP+GOG and SNP+GOG+actinomycin for 8 h and stained with toluidine blue O. Control cells were characterized by a thin primary cell and the presence of large vacuoles (asterisk). The nucleus, located in the centre of the cell, showed a prominent nucleolus (arrow), which stained dark blue with toluidine blue O. A similar morphology was also observed for cells treated with either SNP or GOG at the same concentrations used for the double treatment (data not shown). Cells treated with SNP+GOG showed a granulated cytoplasm (arrowhead) as well as invaginations of the plasma membrane which was detached from the cell wall (arrows). These abnormalities were not visible in cells treated with SNP+GOG+actinomycin (ACT). These cells were similar to control cells. (B) Nuclear morphology of BY-2 cells treated with SNP+GOG and SNP+GOG+actinomycin for 8 h and stained with 4,6-diamino-2-phenylindone (DAPI). Compared with control nuclei, which had a uniform stained chromatin, nuclei of cells incubated with GOG+SNP had a granular appearance. Nuclei of cells treated with actinomycin, as well as SNP or GOG (data not shown), were similar to their control counterparts. Bar=25 μm.
Very similar patterns in label uptake were found at 4 h and 8 h. At both incubation times, uptake of uracil was lower in cells treated with GOG and SNP+GOG, compared with control cells. A similar low uptake value was measured for uridine in SNP+GOG-treated cells (Fig. 4). No statistical differences in uptake among treatments were observed for the other pyrimidine precursors, i.e. orotic acid and thymidine.

**Metabolic fate of pyrimidine precursors**

After 2 h, control cells and cells treated with the NO and H₂O₂ generators (SNP+GOG) were able to incorporate a large fraction of orotic acid and uridine into nucleic acids and nucleotides [UMP+UDPG (UDP-glucose)+UTP] (Fig. 5). Within the nucleotide fraction, a large percentage of radioactivity (more than 80%) was recovered as UTP+UDPG (data not shown). Only a small fraction of label from these two precursors was recovered as degradation products (Fig. 5), mostly CO₂ (data not shown). A different tendency was observed for the other two precursors, uracil and thymidine. In all treatments, salvage of these precursors into nucleic acids and nucleotides was less pronounced (less than 40% of radioactivity recovered in nucleotides and less than 18% found in the nucleotide fraction). A large fraction of radioactivity from both precursors was released as degradation products (Fig. 5).

Significant differences in the utilization of pyrimidine precursors among treatments started to appear at 4 h (Fig. 6). Compared with control cells, cells treated with SNP+GOG showed a reduced incorporation of orotic acid into the nucleic acid fraction, and of uracil into both nucleic acid and nucleotide fractions. In these cells, more than 60% of the radioactivity from uracil was recovered in the degradation products (Fig. 6). Combined applications of GOG and SNP...
also resulted in the increased salvage of thymidine into nucleotides (Fig. 6), mainly TDP+TTP (data not shown).

Major differences in the metabolic fate of pyrimidine metabolites were observed at 8 h. Compared with control cells, the percentage of radioactivity from both uracil and uridine found into nucleic acid and nucleotides was lower in cells treated with SNP+GOG, whereas that released as degradation products, mainly CO₂, was higher (Fig. 7). An opposite tendency was observed for thymidine. The combined application of SNP+GOG increased the utilization of this precursor for nucleotide synthesis and decreased the percentage of radioactivity from thymidine recovered into degradation products (Fig. 7).

**Fig. 6.** Distribution of radioactivity from ¹⁴C-labelled orotic acid (OA), uracil (U), uridine (UR), and thymidine (TR) into nucleic acids (DNA+RNA), nucleotides (UMP+UDPG+UTP for orotic acid, uracil, and uridine; TMP+TDP+TTP for thymidine), and degradation products. Cells were treated for 4 h with SNP, GOG, and SNP+GOG and incubated with the radioactive pyrimidine precursors for the same length of time. Values, expressed as percentage of total incorporation, represent mean values ±SE of three independent experiments. An asterisk indicates values that are significantly different from the control (P<0.01).

**Fig. 7.** Distribution of radioactivity from ¹⁴C-labelled orotic acid (OR), uracil (U), uridine (UR), and thymidine (TR) into nucleic acids (DNA+RNA), nucleotides (UMP+UDPG+UTP for orotic acid, uracil, and uridine; TMP+TDP+TTP for thymidine), and degradation products. Cells were treated for 8 h with SNP, GOG, and SNP+GOG and incubated with the radioactive pyrimidine precursors for the same length of time. Values, expressed as percentage of total incorporation, represent mean values ±SE of three independent experiments. An asterisk indicates values that are significantly different from the control (P<0.01).

**Effects of actinomycin on salvage of uracil, uridine, and thymidine**

At the end of the culture period (8 h), control cells had a significant amount of total radioactivity from both uracil and uridine incorporated in UTP+UDPG (15% from uracil and 18% from uridine) and in UMP (9% from uracil and 16% from uridine) (Fig. 8). These percentages were lowered if cells were treated with SNP+GOG. Inhibition of uracil and uridine utilization for nucleotide synthesis was relieved if these treatments were performed in conjunction with actinomycin (Fig. 8).

Compared to their control counterparts, cells treated with GOG+SNP had a higher ability to salvage thymidine into nucleotides (Fig. 8).
nucleotides (TTP+TDP+TMP). When GOG and SNP were supplied in conjunction with actinomycin, utilization of thymidine for nucleotide synthesis was reduced (Fig. 8).

Enzyme activity

Among the enzymes involved in the salvage of uracil and uridine, UPRT and NPT (UR) increased after 2 h in cells treated with GOG+SNP (Fig. 9). Similar values in the activities of these two enzymes were observed for the other treatments. The activity of URK fluctuated in GOG+SNP-treated cells, reaching its maximum at 4 h (105 pkat mg\(^{-1}\) protein). The specific activity of OPRT, the enzyme participating in the de novo synthesis of pyrimidine precursors (Fig. 1) decreased after 4 h in cells treated with GOG+SNP to values lower than 30 pkat mg\(^{-1}\) protein. Constant values of this enzyme were measured during the course of the experiment for control cells and cells treated with either SNP or GOG (Fig. 9). The combined application of SNP+GOG also resulted in an increased activity of the two thymidine salvage enzymes, i.e. TRK and NPT (TR) (Fig. 1) after 4 h in culture (Fig. 9). Data obtained for cells treated with SNP+GOG+ACT at 8 h support the above findings.

Discussion

Programmed cell death (PCD) is a common physiological process occurring in both animals and plants. In plants, activation of PCD can be an integral component of the normal life cycle, for example, xylem differentiation, tapetal cell degeneration etc., or the result of incompatible plant–pathogen interaction leading to hypersensitive reactions. Due to the participation and interaction of several signal regulatory molecules, including NO and reactive oxygen intermediates (ROI) (Delledonne et al., 2001), studies on PCD are complicated. Difficulties arise when NO and ROI-induced signals, which are related to PCD, have to be separated from those not involved in the process. To overcome this problem, tobacco BY-2 cells have been used in their exponential phase of growth, in which the induction of PCD requires a balance between NO and \(\text{H}_2\text{O}_2\) production, such that high levels of one of the two molecules in the absence of the other are ineffective. This very effective system was developed by de Pinto et al. (2002). In tobacco cells, loss of cell viability (Fig. 2) and the induction of cytological alterations characteristic of PCD, including cytoplasm shrinkage and chromatin condensation (Fig. 3) were promoted by the combined production of NO and \(\text{H}_2\text{O}_2\), effected by applications of SNP+GOG. Additions of either GOG or SNP alone were not able to induce PCD. Furthermore, the effects of SNP+GOG were reversed upon the actinomycin-induced block of transcription and translation, both of which are required for the execution of the death programme (Clark et al., 2000). Therefore, the loss of viability observed in cells treated with SNP+GOG is not due to necrosis, but it is rather regulated by precise mechanisms characteristics of PCD. This is in agreement with previous work, for example, de Pinto et al. (2002).

In both animal and plant cells little is known about the early mechanisms that lead to the commitment of cells to programmed death. Results from this study suggest that an early signal of PCD is represented by alterations in pyrimidine metabolism. These alterations follow a precise pattern: reduced utilization of orotic acid for nucleic acid synthesis and decreased uracil salvage during the first hours, followed by decreased salvage of both uracil and uridine and increased incorporation of thymidine into nucleotides during the late phases of PCD. The reduced incorporation of orotic acid into nucleic acids and the lower utilization of uracil for nucleotide and nucleic acid synthesis in cells treated with GOG+SNP was observed at 4 h (Fig. 6), when a large percentage of cells (68%) was still...
viable (Fig. 2). Poor salvage of uracil also persisted during the subsequent hours in culture (Fig. 7). While OPRT, the enzyme responsible for the conversion of orotic acid to UMP fluctuated during the culture period, the activity of UPRT, the major enzyme involved in uracil salvage was induced by the simultaneous production of NO and H$_2$O$_2$ (Fig. 9). The increased activity of this enzyme may represent an unsuccessful attempt of the SNP+GOG treated cells to increase the rate of the salvage pathway. In these cells, diversion of uracil from nucleic acid and nucleotide synthesis is most likely due to the increased rate of the degradation pathway. Compared with control conditions, degradation of uracil to CO$_2$ and $\beta$-ureidopropionate was promoted by SNP+GOG at 4 h and 8 h (Figs 1, 6, 7). Changes in the balance of the salvage and degradation pathways of uracil metabolism, which have been found to delineate important morphogenic changes during plant development (Ashihara et al., 2001a; Stasolla et al., 2003), may represent an early response of cells undergoing PCD.

As found with uracil, utilization of uridine for nucleotides and nucleic acid production via the salvage pathway was lower in cells undergoing PCD. At 8 h, in fact, less than 55% and 5% of radioactivity from uridine was recovered as nucleic acids and nucleotides in SNP+GOG treated cells, compared with 67% and 22% of control cells (Fig. 7). This decreased salvage activity, is possibly ascribed to the low activity of the main uridine salvage enzyme URK at 8 h (Fig. 9). This enzyme, together with NPT (UR), which appears to be less active in tobacco cells (Fig. 9), has been found to be the main route of uridine salvage in several species (Ashihara et al., 2001a, b). Of interest, alterations in uridine metabolism were also observed during the initial
phases of spruce somatic embryogenesis (Ashihara et al., 2001b), characterized by the programmed death of specific cell types (Filonova et al., 2000).

Alterations in thymidine metabolism also precede PCD in tobacco BY-2 cells. Unlike uracil and uridine, utilization of this precursor for the synthesis of nucleotides, mainly TTP and TDP, was higher in cells treated with SNP+GOG at 4 h and 8 h. The higher level of thymidine incorporation in the nucleotide fraction of these cells is due to the sharp increase in the activity of the two major thymidine salvage enzymes, TRK and NPT(TR), observed after 4 h (Fig. 9). The higher specific activity measured for the latter enzyme at the end of the culture period indicates its prominent role in converting thymidine to TMP. This is in agreement with previous studies (Kameyama et al., 1985).

Based on these observations it is proposed that the alterations in the balance of pyrimidine nucleotide synthesis, resulting in decreased salvage of uracil and uridine and increased salvage activity of thymidine, represent a metabolic switch that establishes proper cellular conditions for the induction of PCD. In particular, some of these events, i.e. decreased utilization of uracil for salvage products, occur very early in the process and can therefore be considered to be an early marker for PCD. Although all these metabolic events are strictly associated with PCD, since they do not occur in the presence of SNP or GOG alone and they are not observed if cell death is inhibited by actinomycin (Fig. 8), their role during PCD is unclear. Alterations in pyrimidine metabolism may result in the imbalance of the endogenous nucleotide pool, which would then lead to mis-incorporation of nucleotides during nucleic acid synthesis and repair and ultimately would activate the death programme. In support of this hypothesis is the observation that perturbation of the endogenous nucleotide pool, effecting by exogenous applications of nucleosides, result in DNA fragmentation and cell death of mouse thymocytes (Kizaki et al., 1988). Similar effects were observed after pharmacological alterations of dNTP pools in BAF3 cells (Oliver et al., 1993), mammary tumor cells FM3A (Yoshioka et al., 1987), and leukaemia cells (Kwok and Tattersall, 1992).

In conclusion, this study has shown that alterations in pyrimidine nucleotide synthesis and utilization represent a metabolic signal that precedes and accompanies the NO and H$_2$O$_2$-induced PCD in tobacco BY-2 cells. Changes in the synthesis and degradation of pyrimidine nucleotides can activate a cellular failure mode which triggers further molecular and cytological processes culminating in the death of the cell.

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