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Cell death induced by sodium nitroprusside and hydrogen peroxide in tobacco BY-2 cell suspension

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Abstract

The interplay between nitric oxide (NO) and reactive oxygen species can lead to an induction of cell death in plants. The aim of our work was to find out if cyanide released from sodium nitroprusside (SNP; a donor of NO) could be involved in the cell death induction, which is triggered by SNP and H_2O_2. Cell suspension of *Nicotiana tabacum* L. (line BY-2) was treated with 0.5 mM SNP, 0.5 mM potassium ferricyanide (PFC; analogue of sodium nitroprusside which can not release NO) and/or by 0.5 mM glucose with 0.5 U cm^{-3} glucose oxidase (GGO; a donor system of H_2O_2). The cell death was induced only by combination of SNP and GGO. Thus cyanide released was not involved in the induction of cell death. However, SNP showed toxic effect because of decrease in activities of intracellular oxidoreductases and esterases. The cell death caused by SNP and GGO occurred within 12 h. During cell death either length or width of the cell increased. Central vacuole was formed in 20 to 40 % of cells. Most of the dead cells showed a condensed cytoplasm. Two hallmarks of programmed cell death (PCD), chromatin condensation and blebbing of nuclear periphery, were observed. However, oligonucleosomal fragmentation of DNA, another hallmark of PCD, was not detected.

Additional key words: *Nicotiana tabacum*, nitric oxide, potassium ferricyanide, programmed cell death.

Introduction

Nitric oxide has been recently recognized to play a significant role in plants, which are capable of both sensing of exogenous NO and its synthesis. Nitric oxide has been shown to be involved in growth and maturation and senescence (Leshem 2000). It also plays a crucial role in the stress physiology of plants (Buchanan *et al.* 2002). During biotic stress NO acts like a signalling molecule, which regulates the defence reaction against pathogens (Delledonne *et al.* 2002). Trials with pathogen treated plants have proven that NO can trigger the cell death in association with reactive oxygen species (ROS), however, the ratio of steady state concentrations of NO and ROS has to be within a certain range. This finding is supported by experiments with artificial donors of NO and ROS (Delledonne *et al.* 2002).

The most popular donor of NO used by plant biologists is sodium nitroprusside (SNP - $Na_2[Fe(CN)_5NO]$ disodium nitrosoferricyanide; *e.g.* Delledonne *et al.* 1998, Pedroso *et al.* 2000, de Pinto *et al.* 2002). In addition to NO release, this compound can generate highly toxic cyanide (Wang *et al.* 2002) due to presence of the ferricyanide moiety. Cyanide can strongly affect the metabolism of cells because of its high affinity to metal cofactors of many enzymes, particularly it can inhibit cytochrome *c* oxidase in the respiratory chain (Grossmann 1996). On the other hand, cyanide is a by-product of ethylene biosynthesis. Thus plants are able to sustain and detoxify cyanide produced under physiological conditions (Grossmann 1996, Borecký and Vercesi 2005).

The aim of our work was to find out if cyanide released from SNP takes a part in the induction of cell death by SNP and hydrogen peroxide (H_2O_2). Furthermore we were interested in biochemical and morphological characteristics of the cell death process induced by the above-mentioned compounds.

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Abbreviations: BAP - N^6-benzylaminopurine; CTAB - hexadecyltrimethylammonium bromide; FDA - fluorescein diacetate; GGO - glucose with glucose oxidase; MTT - methylthiazolyltetrazolium bromide; PCD - programmed cell death; PFC - potassium ferricyanide; PI - propidium iodide; ROS - reactive oxygen species; SNP - sodium nitroprusside.

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Materials and methods

BY-2 cell suspension: The tobacco (*Nicotiana tabacum* L. cell line BY-2; Nagata *et al*. 1992) cell suspension (20 cm^3 in 50 cm^3 Erlenmayer flasks) was maintained in liquid Murashige and Skoog medium, which was supplemented by 30 g dm^{-3} sucrose, 0.2 g dm^{-3}) KH_2PO_4, 1 mg dm^{-3} thiamine and 0.2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (Nagata *et al*. 1992). Culture flasks were placed in a shaker (model *LT-W*, *Kühner*, Birsfelden, Switzerland) in dark. The temperature and the agitation were set to 27 °C and 135 min^{-1}, respectively. The subcultivation was carried out twice a week. The 3-d-old culture with cell density approximately 2×10^6(cells) cm^{-3} was used for experiments.

Induction of cell death: Aliquots of the cell suspension (20 cm^3) were treated with addition of SNP to the final concentration of 0.5 mM, GGO to the final concentration of 0.5 mM glucose and 0.5 U cm^{-3} glucose oxidase (low in catalase, *Sigma*, Prague, Czech Republic), respectively, and by combination of SNP and GGO (De Pinto *et al*. 2002). As a parallel PFC was used instead of SNP. All solutions added into the suspension were sterilised by membrane filter (*Puradisc 25 AS*, 0.2 μm, *Whatman*, Clifton, NJ, USA). Glucose oxidase and SNP solutions were prepared immediately prior to use.

Benzylaminopurine treatment: Membrane sterilised (*Puradisc 25 AS*) stock solution of N^6-benzylaminopurine (BAP) in 0.1 M KOH was added into cell suspension up to the final concentration of 100 μM. Small shift of pH because of hydroxide addition was corrected by sterile 0.1 M HCl.

Viability assay: The viability was detected by modified staining with fluorescein diacetate (FDA) and propidium iodide (PI) (Jones and Senft 1985). The sample of cells (0.01 cm^3) was diluted up to 0.05 cm^3 with fresh medium and incubated with FDA (1 μg cm^{-3}) and PI (20 μg cm^{-3}) for 5 min. The ratio of dead (red stained) and viable (green stained) cells was evaluated by the fluorescence microscope equipped with wide range UV excitation optics (a set of optical filters *U-MWU*, *Olympus*, Prague, Czech Republic). At least 400 cells were counted.

Cell density assay: Cell density (number of cells per cm^3) was determined using a *Fuchs-Rosenthal* haemocytometer (*Glaswarenfabrik Karl Hecht KG*, Sondheim, Germany).

Intracellular esterase assay: Intracellular esterases were determined by FDA assay (Víteček *et al*. 2004, 2007, Petřek *et al*. 2005). Briefly, 0.5 cm^3 of cell suspension was washed three times with 50 mM potassium phosphate buffer (pH 8.7) and homogenised with extraction buffer (250 mM potassium phosphate, 1 mM dithiothreitol, pH 8.7) in a *Potter-Elvehjem* homogeniser (*Kavalier*, Sázava, Czech Republic) placed in an ice bath. Cell debris was removed by centrifugation (10 000 *g*, 4 °C, 15 min). Aliquot of the cell extract (0.005 - 0.05 cm^3) was complemented to the final volume of 1 cm^3 by 1 M potassium phosphate buffer (pH 8.7). The reaction was started by addition of FDA to the final concentration of 5 μM. After 15 min of incubation at 45 °C, the released fluorescein was assessed fluorimetrically (spectrofluori-meter *RF 551*, *Shimadzu*, Duisburg, Germany, excitation at 490 nm and emission at 514 nm). One unit of esterases liberates 1 μmol of fluorescein per minute under specified conditions.

MTT assay: Methylthiazolyltetrazolium bromide (MTT) was added to sample of the cell suspension (0.5 cm^3) to the final concentration of 0.5 mg cm^{-3}. Cells were collected by centrifugation (360 *g*, 25 °C, 5 min) after 1 h of incubation in the shaker (120 min^{-1}) at 25 °C. Dark purple formazan, formed from MTT, was extracted by 1.5 cm^3 of isopropanol for 30 min. Extract was clarified by centrifugation (10 000 *g*, 25 °C, 10 min). Its absorbance was measured at 570 nm.

Nuclear ultrastructure: Cells were fixed by mixing of cell suspension with PEM buffer [1:1, 100 mM piperazine-1,4-bis(2-ethanesulfonic acid), pH 6.9, 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetra-acetic acid, 10 mM $MgCl_2$] containing 4 % formaldehyde. Then, the cells were washed three times with PEM buffer (Callard *et al*. 1996) after 30-min fixation. Finally they were resuspended in PEM buffer supplemented by 0.1 % (v/v) of *Triton X100* and 1 μg cm^{-3} *Hoechst 33285*. Cell nuclei were observed with the fluorescence microscope *AX 70* (*Olympus*, Prague, Czech Republic) using a wide range UV excitation (set of optical filters *U-MWU*). At least 200 nuclei were observed in order to obtain ratio of a particular shapes of nuclei.

Image analysis: The cell suspension was incubated with FDA (1 μg cm^{-3}) for 5 min and observed with the fluorescence microscope *AX 70* using wide range UV excitation optics (see above) or a phase contrast mode.

Images in phase contrast mode were processed with software *Image Pro* (version *1.3*, *Sony*, San Diego, USA). Cell size was obtained using the mode based on a rectangle drawn around the cell. The longer side of the rectangle was regarded to be cell length. Alt least 50 randomly chosen cells were analysed in each specimen.

Assay for DNA fragmentation: DNA was isolated and purified by modified CTAB method (Murray and Thompson 1980). Briefly, samples of cells (100 - 500 mg, stored at -80 °C) grounded to a fine powder in liquid nitrogen was mixed with extraction buffer [2 % cetyltrimethylamonium bromide (CTAB), 2 % poly-

vinylpyrrolidone, 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl, pH 8.0]. After melting, the mixture was incubated at 65 °C for 40 min. Proteins were removed by chloroform-phenol extraction (chloroform:phenol 9:1). DNA was precipitated from the purified extract by addition of ice-cold isopropanol (solution:isopropanol 1:1). Pellet of DNA was dissolved in 0.2 cm^3 of TE buffer. The solution was incubated with RNAse (final concentration 40 μg cm^{-3}) at 65 °C for 10 min to digest RNA. The procedure was repeated from chloroform-phenol extraction up to dissolution of DNA. The concentration of DNA was estimated with fluorimetric assay using *Hoechst 33258* (Labarca and Paigen 1980). Fragmentation of DNA was evaluated by standard electrophoresis on the 1 % Tris-acetate gel (described elsewhere).

Data analysis: Data were processed and analysed using *Microsoft Excel* (version *9.0*). If not otherwise indicated data are given as mean ± standard error of mean.

Results

SNP posses the NO-specific effect in cell death induction: Tobacco BY-2 cells were treated with 0.5 mM SNP (a donor of NO), 0.5 mM PFC - an analogue of SNP that can not release NO, GGO (0.5 mM glucose with 0.5 U cm^{-3} glucose oxidase) - a system for H_2O_2 generation, and by combinations of SNP with GGO and PFC with GGO as well. During the 12-h period the cell viability was decreased only by SNP with GGO (Fig. 1*A*). Dying of the cells induced by SNP with GGO was observed alraedy after 4 h and all cells were dead at the end of experiment.

The MTT assay revealed that control (untreated) cells exhibited a continual increase of oxidoreductase activity (approximately 5 % per hour, in correlation with cell density). As for treated cells, the oxidoreductase activity decreased quickly (10 min) and did not show marked changes after 2 h of the treatment except the cells treated with SNP and GGO, where we did not determine any oxidoreductase activity after 8 h (Fig. 1*B*).

The esterase activity of control cells, assayed by the FDA test, increased during the experiment (approximately 8.3 % per hour, in correlation with cell density) while treated cells showed a decrease of esterase activity. There were no statistical differences (α 0.05) among parallels treated with SNP, PFC, GGO and PFC with GGO. The esterase activity of the cells treated with SNP and GGO gradually decreased almost to zero at 12 h (Fig. 1*C*). There were no changes in total cell density in all treated parallels during the experiment.

Cell death characteristics: Size of cells treated with SNP and GGO was assayed by image analysis. The cell length increased from 47 ± 7 μm up to 115 ± 20 μm at the end of experiment and cell width from 33 ± 4 μm to 61 ± 7 μm. The more detailed observation of the cell morphology was carried out after 6 h of treatment. Untreated cells in the exponential growth phase had a rectangular shape typical of line BY-2 and contained several transvacuolar strands (Fig. 2*A*). Malformed cells (less than 5 %) appeared in all parallels except the control one. Their shape was deformed and contained an extreme amount of vacuoles and transvacuolar strands (Fig. 2*A*). Moreover, substantial number of cells (about 20 to 40 %) treated with SNP with GGO showed a reduced number of

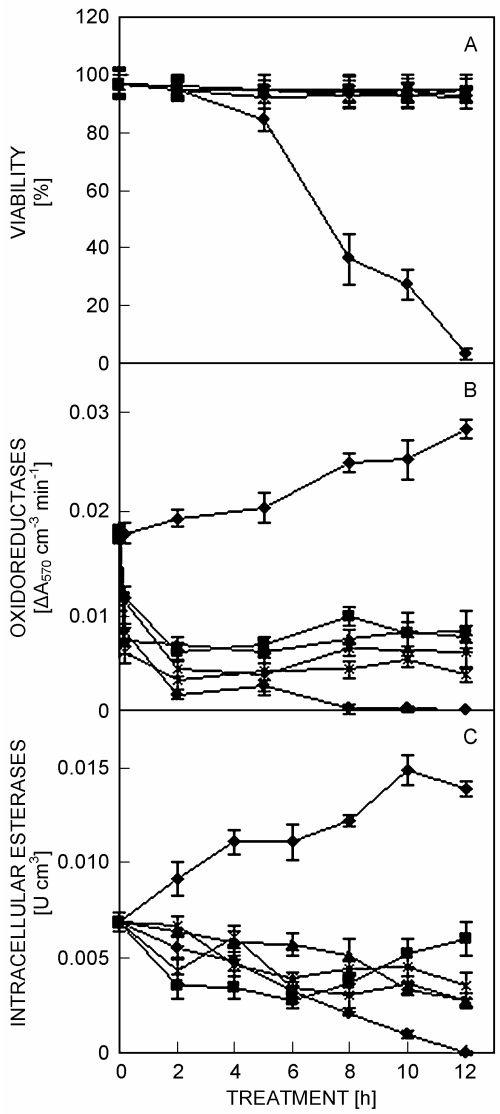

Fig. 1. Time course of viability (*A*), oxidoreductase activity (*B*) and intracellular esterase activity (*C*) in the suspension treated with GGO (0.5 mM glucose with 0.5 U cm^{-3} glucose oxidase; *squares*), 0.5 mM PFC (*triangles*), 0.5 mM SNP (*crosses*), PFC with GGO (*asterisks*), SNP with GGO (*circles*), and in the untreated suspension, which served as a control (*rhombs*). Means ± SE, $n = 3$.

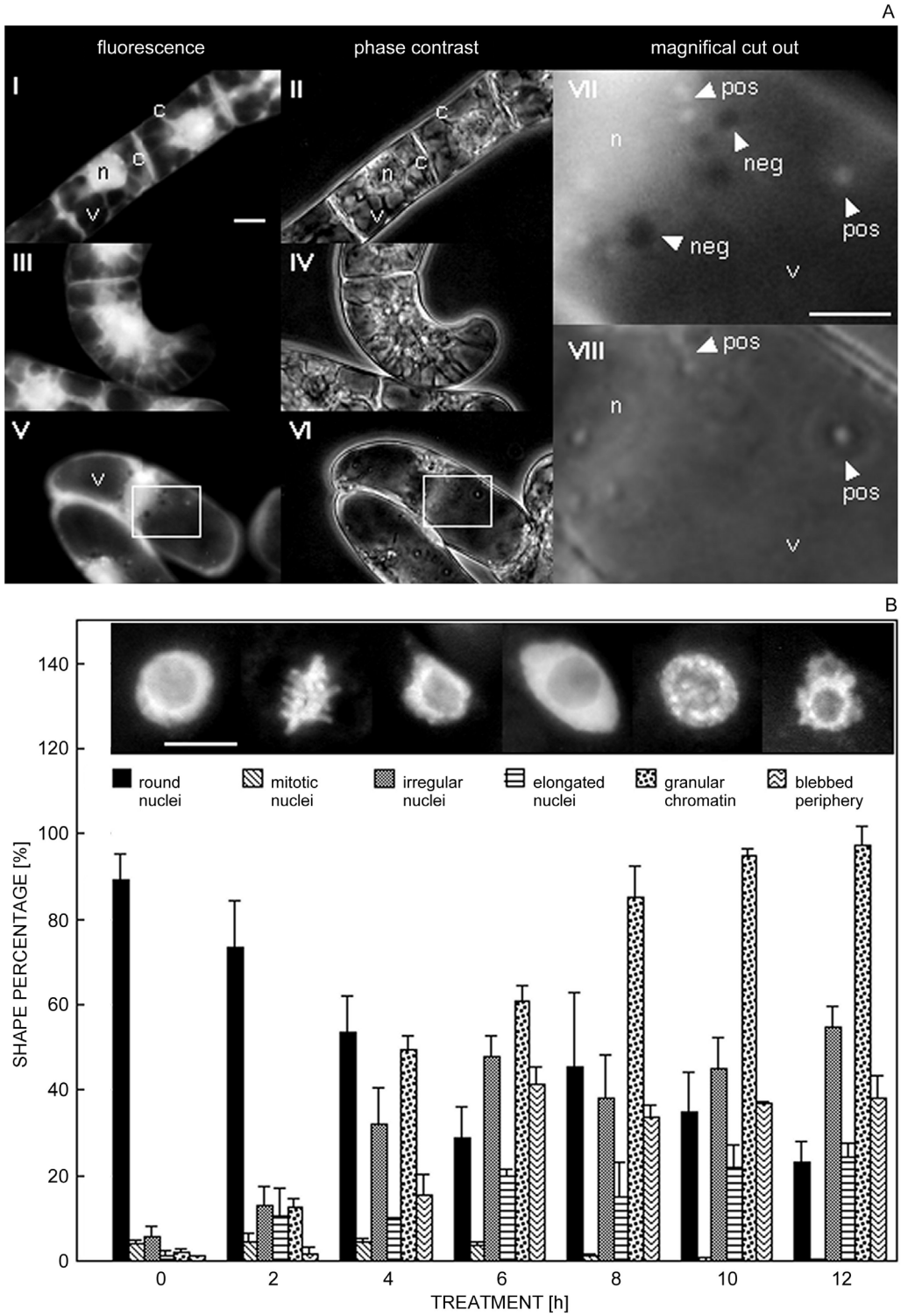

Fig. 2. *A* - Influence of 0.5 mM SNP, 0.5 mM PFC , GGO (0.5 mM glucose with 0.5 U cm^{-3} glucose oxidase), PFC with GGO, and SNP with GGO on ultrastructure of BY-2 tobacco cells visualised by fluorescence (FDA staining) and phase contrast microscopy after 6 h of treatment: control cells (I, II), non-specific malformations caused by SNP, PFC, GGO, and PFC with GGO (III, IV), cells treated with SNP with GGO (V, VI) and the magnified cut out of images V and VI, respectively. Symbols indicate cytoplasm (c), nucleus (n), vacuole (v), negatively (neg) and positively (pos) stained vesicles. *B* - Shapes of nuclei occurring in BY-2 cells when treated with SNP and GGO and time dependent changes in percentage of these shapes. Means ± SE, $n = 3$. Images in upper part show typical manifestations. *Bar* = 10 μm.

vacuoles or even a single central vacuole. Small vesicles negatively stained with FDA were localized in the perinuclear cytoplasmic zone. There were FDA positively stained granules in the cytoplasm as well. Unlikely the negatively stained vesicles they were visible in the phase contrast (Fig. 2*A*). Shrunken cytoplasm was typical of more than 70 % of dead cells (not shown).

In cells treated with SNP and GGO, nuclei had mostly regular rounded shape with uniformly stained chromatin and clearly visible nucleoli at the beginning of the experiment. About 4 % of the mitotic nuclei and about 6 % of misshapen (irregular and prolonged) nuclei were present. During the treatment mitotic nuclei disappeared. On the other hand the ratio of misshapen nuclei and nuclei containing granular chromatin or having a blebbed periphery gradually increased. The first manifestations of the granulation process were observed after 2 h of the treatment, 50 % of nuclei became granulated after 4 h, and after 8 h, almost all of nuclei contained granular chromatin. Deformation of nuclei showed about the same onset, but it reached the maximal value earlier and it was not so extensive (about 65 %). Also blebbing of the nuclear periphery occurred. Unlikely the granulation, increased percentage of these nuclei was observed after 4 h (about 15 %) and reached about 40 % at the end of experiment (Fig. 2*B*).

In addition, we focused on the integrity of DNA during the cell death. No degradation of DNA in cells treated with SNP and GGO has been observed. Cells treated with 100 μM BAP for 48 h were used as a positive control for the procedure verification (Fig. 3), because cytokinins are known to induce cell death accompanied by DNA cleavage to oligonucleosomal fragments (Carimi *et al.* 2003, Mlejnek *et al.* 2003).

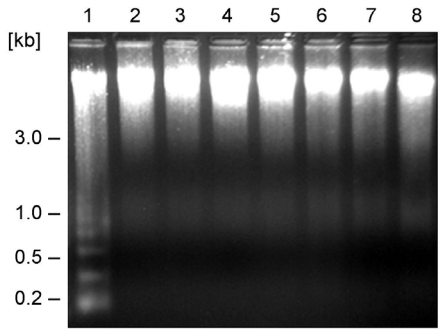

Fig. 3. Detection of DNA integrity on 1 % agarose gel during cell death induced by SNP with GGO. *Lane 1* - positive cotrol, treatment with 100 μM BAP for 48 h. *Lanes* 2 to 8 - samples taken after 0, 2, 4, 6, 8, 10, 12 h of treatment with 0.5 mM SNP with GGO.

Discussion

Recently published papers have shown that NO and ROS may induce the cell death in plants. These findings are also supported by experiments with artificial sources NO and ROS (Delledonne *et al.* 1998, Pedroso *et al.* 2000, de Pinto *et al.* 2002, Faoro and Iriti 2005). On the other hand, NO may act as antioxidant and delay the cell death process (Beligni *et al.* 2002) or alleviate plants from drought stress (Tian and Lei 2006). One of the most popular donors of NO used by plant biologists is SNP due to convenient properties. The adverse feature of this compound consists in its ferricyanide moiety because during decomposition of SNP cyanide anions could be released in addition to NO (Wang *et al.* 2002). The decomposition of SNP is enhanced by lower pH (5.0 and below) (Greenwood and Earnshaw 1998). Taking pH of plant cell suspension into consideration (5.2 to 5.8, pH of the BY-2 cell suspension) the cyanide releasing may play a crucial role.

In order to address this question, the same concentration of PFC an analogue of SNP, which cannot release NO, was used together with GGO like a control. During the experiment, the combination of PFC with GGO did not induce any decrease in cell viability compared to SNP with GGO (Fig. 1*A*). Thus released cyanide from the ferricyanide moiety of SNP should not be involved in the induction of cell death by SNP with GGO. Further experiments have revealed that all active compounds in the suspension (H_2O_2 generated by GGO, SNP and PFC) are capable of suppressing MTT detectable oxidoreductase activity (Fig. 1*B*). A wide range of oxidoreductases within the cell reduces MTT (Berridge and Tan 1993). Thus, we were interested in the issue if the compounds that can penetrate through cytoplasmic membrane can influence MTT reduction. H_2O_2 is a small molecule without any charge therefore it can permeate easily through biomembranes. PFC is reported to be membrane impermeable (Golovina *et al.* 1997). Based on analogy between SNP and PFC, SNP can be also regarded as membrane impermeable. In spite of that, oxidoreductases were affected immediately after the start of the experiment. Releasing of cyanide from SNP and PFC is the possible reason how they can influence the oxidoreductase activity, because cyanide moiety as HCN (a non-charged small molecule) can permeate the cell membrane. The effect of SNP and PFC on the oxidoreductase activity was similar, which supported our above-mentioned explanation. The simultaneous action of SNP and H_2O_2 on the oxidoreductase activity was the most substantial one. The oxidoreductase activity was negligible after 8 h of treatment, even though there were around 40 % of viable cells. This indicated destruction of the redox metabolism in the remaining viable cells.

In addition to MTT detectable oxidoreductases, which represent redox metabolism, esterase activity was determined as well. Esterases were shown to be a suitable marker for *in vitro* viability assay (Steward *et al.* 1999, Amano *et al.* 2003). Recently we have demonstrated that

the activity of intracellular esterases can be considered as a marker of number of viable cells in cell suspensions (Víteček *et al.* 2004, 2005). Because of the low substrate specificity (Bornscheuer 2002), esterases can be also regarded as representatives of the family of hydrolytic enzymes. Therefore a decrease of intracellular esterase activity give an idea about the state of hydrolytic metabolism, even if there is no change of viability detected by double staining by FDA and PI or by dye exclusion test. It was found out that intracellular esterase activity of the untreated cells as well as their oxidoreductase activity exhibited a major increase, which correlated with the growth of the cell suspension. The results from other parallels obtained by the esterase measurement indicated that hydrolytic metabolism was also affected by SNP, PFC and H_2O_2. However, the extent of metabolic suppression was not as intensive as in the case of oxidoreductase activity (Fig. 1*C*).

Based on the obtained results, we can summarize that enzymes involved in redox metabolism are more sensitive to cyanide because they often contain metal cofactors, which can bind cyanide easily (Mathews *et al.* 2000) and H_2O_2 as an oxidant may cause a decrease of intracellular reducing equivalents.

As we mentioned above, coordinated action of SNP and H_2O_2 induces the cell death in a suspension culture (Delledonne *et al.* 1998, de Pinto *et al.* 2002), but the mechanism is not clear yet. It could be because of the disruption of pyrimidine nucleotide metabolism (Stasolla *et al.* 2004). This cell death is accompanied by cytoplasm shrinkage and nuclear granulation (De Pinto *et al.* 2002, Stasolla *et al.* 2004), which are hallmarks of programmed cell death (PCD) (Havel and Durzan 1996a,b, Van Barleen *et al.* 2004). We focused on changes of cell content during the process of cell death induced by SNP and H_2O_2 as well as detailed study of nuclear changes.

It was found that during the cell death the size of cells changed. Length of cells as well as their width increased significantly. Other experiments concerning BY-2 line showed that cells may either prolong, *e.g.* by treatment with sublethal concentration of Cd^{2+} (Kuthanová *et al.* 2004) or contract, *e.g.* by cytokinins (Mlejnek *et al.* 2003). In both cases it was slow process (range of days), which contrasts with the rapid changes in BY-2 cells treated with SNP and GGO. We observed that SNP (as well as PFC) or GGO induced formation of extensive number of transvacuolar strands and malformation of the cell shape in a small number (about 5 %) of the cells (Fig. 2*A*). That is why it can be regarded as a non-specific response of BY-2 cells to stress. Similar effect was found in BY-2 cells treated with 50 μM Cd^{2+} by Kuthanová *et al.* (2004), but it was rather a specific response to heavy metal stress. In addition, the influence of combination of SNP and GGO, which lead to the cell death, was accompanied by a formation of central vacuole in 20 to 40 % of cells. Such organelle can be involved in the degradation of cellular content (Rogers 2005). Central vacuole also emerged in BY-2 cells under sucrose starvation, however it's lytic function has not been confirmed (Moriyasu and Ohsumi 1996). Positively and negatively FDA stained vesicles appeared in the cytoplasm but only positively FDA stained vesicles were visible in the phase contrast (Fig. 2*A*). This indicates that they contained solid particles unlike the negatively FDA stained ones. Considering their size and shape, positively stained vesicles may be stress-induced derivatives of endoplasmic reticulum. (Matsushima *et al.* 2002). Cytoplasm condensation, which has already been observed in dead cells when treated with SNP and GGO (De Pinto *et al.* 2002, Stasolla *et al.* 2004) is regarded as one of the hallmarks of PCD (Havel and Durzan 1996a,b).

Furthermore, SNP with GGO caused extensive changes of nuclei (Fig. 2*B*). There were observed deformations of the nuclei (irregular and prolonged ones) as well as two hallmarks of PCD: chromatin granulation and blebbing of the nuclear periphery (Havel and Durzan 1996a,b). Unlike other works concerning PCD in BY-2 culture (Mlejnek and Procházka 2002, Houot *et al.* 2001) we did not observe any formation of apoptotic bodies or condensation of chromatin at nuclear envelope. Virtually all nuclei underwent the granulation process regardless of changes of their shape. The granulation of chromatin preceded the cell death hence it seems to be an active process in living cells. Deformation of the nuclei started together with their granulation but was not so extensive. Changes of the shape of nucleus or switching between condensed and non-condensed state is likely due to posttranslational modifications of DNA scaffold proteins (Berardi *et al.* 2004). This suggests that the scaffold proteins are strongly affected during the PCD process induced by SNP and GGO. Probably, it goes *via* proteolytic enzymes that are associated with several types of plant PCD (Woltering *et al.* 2002). The blebbing of the nuclear periphery showed a later onset but also preceded cell death, hence it was an active process in cells.

Such fast PCD as we observed sharply contrasts with data obtained on treatment with other PCD inducing agents like cytokinins (Mlejnek and Procházka 2002, Carimi *et al.* 2003) and heavy metals (Fojtová and Kovařík 2000, Kuthanová *et al.* 2004) when the cell death showed a progress over several days. Simultaneous synthesis of NO and ROS is mostly attributed to the plant-pathogen interaction which can lead to the fast cell death (Delledonne *et al.* 2001). It has been shown that another hallmark of PCD, degradation of DNA into oligonucleosomal fragments, may be also present in elicitor induced PCD (Sasabe *et al.* 2000). The PCD induced by SNP with GGO was not accompanied by DNA laddering (Fig. 3). In this case probably large DNA fragments were formed. The larger DNA fragments are also being recognized in PCD (Bortner *et al.* 1995). Although the concerted synthesis of NO and ROS is capable of cell death induction, it need not necessarily trigger all processes involved in the induced cell death.

We showed that SNP with GGO induced cell death specifically by release of NO. Thus the ferricyanide moiety, which can release cyanide, was demonstrated to be inactive in the induction of cell death. However, we

proved that it negatively influences the cells, because it brought about the rapid decrease of oxidoreductase activity. The cell death induced by SNP and GGO occurred within 12 h. It was accompanied by increase of cell size, formation of central vacuole in significant proportion of cells, cytoplasm shrinkage and chromatin condensation as well as blebbing of nuclear periphery typical of PCD, but not by fragmentation of DNA, which is also regarded to be a hallmark of PCD.

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