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MASTOPARAN-INDUCED CELL DEATH SIGNALLING IN CHLAMYDOMONAS REINHARDTII

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ABSTRACT
The present study was focused on the elucidation of stress-induced cell death signaling events in the unicellular alga Chlamydomonas reinhardtii exposed to treatment with wasp venom mastoparan. By applying pharmacological approach with specific inhibitors, we have investigated the involvement of ethylene, nitric oxide and lipid signaling in MP-treated C. reinhardtii. Superior sensitive laser-based detectors were used for real-time measurement of trace amounts of ethylene and nitric oxide. The morphological features in the cells undergoing programmed cell death were detected by using laser-scanning confocal microscopy. Data showed that mastoparan induces programmed cell death in C. reinhardtii 137 C(+) that is associated with phospholipid signaling, including phospholipases C and D, ethylene and nitric oxide and, the dead cells express apoptotic-like cellular disintegration, involving cytoplasm shrinkage and condensation of the nucleus.

Keywords: Cell death, Chlamydomonas reinhardtii, Ethylene, Nitric oxide, Phospholipids

Introduction
Programmed cell death (PCD) is highly organized process of cellular suicide activated developmentally and in response to diverse biotic and abiotic insults. PCD machinery operates in a crosstalk of various signaling pathways and molecules. Morphological hallmarks of PCD are cell and cytoplasm shrinkage, condensation of the nucleus, DNA fragmentation. Mastoparan (MP) is a tetracadecapeptide occurring in wasp venom. It activates trimeric G-proteins by mimicking the intracellular α-helix loops of trans-membrane receptors (13). There is evidence that trimeric G-proteins are also present in plants (16). It has been shown that MP can stimulate numerous responses, including the activation of phospholipase A2 (PLA), C and D (9) and to induce plant defence responses (14). It has been shown that MP induces PCD in tomato cell culture (12), but limited information is available on the signaling in MP-induced cell death in C. reinhardtii.

Ethylene is a gaseous phytohormone that participates in many aspects of plant developmental processes (2) and is illustrated to accompany cell death in response to environmental cues, chemicals and at plant-pathogen interactions (6, 11). Nitric oxide (NO) is a bioactive molecule that regulates diverse physiological processes (7). NO has been documented as a component of the signal-transduction network that connects plant responses to primary signals, including hormones, elicitors of defence responses or abiotic stresses (1). Depending on the model system, nature and severity of applied stress, NO can play a dual role as a potent anti-apoptotic agent and also as PCD inducer (21).

Representing single cells and entire organism, the unicellular green alga Chlamydomonas reinhardtii is a suitable model system to study the cell death. Exposure of C. reinhardtii to UV has been shown to induce apoptotic-like PCD features such as TUNEL positive nuclei and DNA laddering (15) and autophagic form of PCD has been observed in response to treatment with rapamycin (3).

The potency of the wasp venom mastoparan to stimulate cell death in the unicellular green alga Chlamydomonas
reinhardtii 137 C (+) was examined. By applying pharmacological approach with administration of specific inhibitors, we have investigated the involvement of lipid signaling, ethylene and nitric oxide in MP-treated C. reinhardtii. Superior sensitive laser-based detectors were used for real-time measurement of trace amounts of ethylene and NO.

Materials and methods

Chlamydomonas growth and culture conditions

C. reinhardtii wild type strain 137 C (+), provided by the collection of Assoc. Prof. Stephka Chankova, Central Laboratory of General Ecology, BAS, Sofia, Bulgaria, was maintained on solid Sager-Granick (SG) medium (10) in glass tubes under continuous illumination at light intensity of 90 μmol. m⁻². s⁻¹, at 25°C. Vegetative cells of C. reinhardtii were grown in liquid Tris Acetate Phosphate (TAP) medium (10), in orbital shaker at 100 rpm under the same light and temperature conditions. Cultures with cell density of 3.10⁶ cells/ml at the late logarithmic phase of growth were used for cell death experiments and gas measurements.

Chemical treatments

For induction of cell death, MP (1 - 30 μM) was applied to 10 ml of suspension culture in 30 ml bottles with gas tight screw-caps. To allow the penetration into the cells, the chemicals were introduced 40 min before the treatment with MP. In general, the used substances were tested in a range of concentrations (from nM to mM) with and without the addition of 1 μM MP. Lowest concentrations giving significant modulation of MP-induced cell death are presented. Lyso phosphatidyl ethanolamine (L-PEA), 1-(6-((17β)-3-methoxyestra-1,3,5(10)-tri-en-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122) were dissolved in dimethyl sulfoxide (DMSO), final solvent concentration in treated cell culture 0.1% v/v. DMSO was tested alone in the indicated concentration and no effect on cell viability and on the amount of measured gases was detected. MP was dissolved in water.

Cell death determination

Cell death in MP-treated suspension was determined at intervals from 0 to 120 h or 24 h after the chemical treatments, after staining with 0.002% fluorescein diacetate (FDA, green fluorescence visible in the viable cells only). Cell death was calculated as a percentage of dead cells to the total number of cells. Propidium iodide (PI) was used for visualization of dead nuclei. Cell counting was executed by using fluorescence microscope (Nikon Eclipse, Vienna, Austria, TS 100, filter B-2A, exciter 450-490, DM 505, BA 520).

Ethylene production measurement

Ethylene production was measured using a sensitive laser-based ethylene detector (type ETD-300, Sensor Sense B.V., Nijmegen, the Netherlands) in combination with a gas handling system. The ETD-300 is a state-of-the-art ethylene detector based on laser photoacoustic spectroscopy (4, 5). Six glass cuvettes (30 ml volume) containing 10 ml cell suspension with density 3.10⁶ cells/ml were used per experiment. For allowing the detection of low amounts of ethylene, accumulation time per cuvette was adjusted to 100 min and after that period each cuvette was flushed with air at constant flow of 2 l/h.

Nitric oxide measurement

Nitric oxide production was measured using a Quantum Cascade Laser (QCL) - based spectrometer, equipped with an astigmatic multi-pass absorption cell for wavelength modulation spectroscopy on NO (5). For on-line concentration measurements and data analysis, LabVIEW program (National Instruments, USA) was used (5). Three glass cuvettes (30 ml volume) containing 10 ml cell suspension with density 3.10⁶ cells/ml were used per experiment. Accumulation time per each cuvette was 75 min and after that period each cuvette was flushed with air at flow of 1.66 l/h.

Imaging

Fluorescent and bright field images of Chlamydomonas suspension cells were collected 24 h after MP treatment, by using the transmission channel and the 488 nm excitation line of the argon laser of a TCS SP2 AOBBS confocal laser scanning microscopy system (Leica-Microsystems GmbH, Mannheim, Germany) mounted an inverted DM IRE2 microscope and, by fluorescent microscope (Nikon Eclipse, Vienna, Austria, TS 100, filter B-2A, exciter 450-490, DM 505, BA 520) equipped with Nikon DXM 1200 digital camera.

Results and Discussion

MP effect on cell death in suspension cells of C. reinhardtii occurred in a concentration dependent manner with 50 % dead cells at 1 μM MP and reached 100% in 24 h at 30 μM MP (Fig. 1A). In further experiments, cells were routinely treated with 1 μM MP. The morphology of vital and dead cells was examined by fluorescent and confocal microscopy.
Following FDA staining living cells show green fluorescence (Fig. 1C), whereas the dead cells do not (Fig. 1D). The dead cells were identified after PI staining of the dead nuclei (Fig. 1E). Non-treated control cells showed a normal placed nucleus, intact cytoplasm and well defined membranous organelle morphology (Fig. 1B and C). In contrast, MP-treated dead cells revealed typical PCD morphology. They underwent progressive shrinkage in size, increased vacuolization, cytoplasm shrinkage and separation from the wall and nucleus condensation (Fig. 1D and E). PCD symptoms were similar to the described in Cd-treated tomato cells (11). This indicates that MP-induced cell death in *C. reinhardtii* resembles features of chemical-induced PCD in other plant systems.

**Fig. 1.** Dose effect of MP on cell death in suspension cultured cells of *C. reinhardtii* (A) and confocal laser scanning microscopic images (B-E) showing morphological changes in MP-treated cells of *C. reinhardtii*: B. living cell and D. dead cell—transmission light imaging; C - living cell (FDA staining); E - PI staining – the dead cells show cytoplasm shrinkage, vacuolization and condensation of the nucleus. The images were collected 24 h after the application of 1 μM MP. Cw, cell wall; cyt, cytoplasm; nu, nucleus; chl, chloroplast; v, vacuole; fl, flagellum. Error bars in A represent SEM (n = 1).

To elucidate the signal transduction pathways involved in MP-induced cell death, inhibitors specific to phospholipid signalling were applied. Activated PLC hydrolysis phosphatidylinositol 4,5-biphosphate into two second messengers: diacylglycerol and inositol 1,4,5-triphosphate (IP3), which evokes changes in intracellular calcium concentrations. The PLC inhibitor U-73122 reduced twice MP-stimulated cell death (Fig. 2). Activated PLD produces phosphatidic acid (PA), by hydrolyzing structural lipids. 1-Butanol may substitute for water in the PLD-mediated formation of PA giving rise to the production of the inactive product phosphatidylbutanol and thereby inhibiting PA-dependent responses (8). The administration of 0.1 % 1-butanol significantly reduced MP-triggered cell death. 2-Butanol does not serve as an alternative substrate for PLD and was used as a negative control and, did not affect MP-induced cell death. L-PEA is a product of the PLA2 catalyzed hydrolysis of membrane glycerophospholipids and is known to inhibit PLD. L-PEA decreased MP-triggered cell death (Fig. 2). It has been shown that treatment of biflagellate green alga Chlamydomonas with MP activate PLC and PLD and induce flagellar excision (17, 19). The participation of PLC and PLD has been established in Cd-challenged tomato cell culture after application of several inhibitors of PLC (U-7312 and neomycin) or PLD (L-PEA and 1-butanol) (20). Our results indicate that both PLC and PLD signalling pathways are involved in MP-induced cell death in Chlamydomonas and may affect further signalling trough increased levels of IP3, calcium (data not included) and perhaps other PLC and PLD-derived second messengers which activate a variety of different enzymes including protein and lipid kinases, among them IP-3-kinases, MAPKs and protein kinase C that have been associated with PCD in animal systems (18).

**Fig. 2.** Effect of inhibitors of lipid signalling on MP-induced cell death. Cell death was determined after FDA staining of the living cells 24 h after treatment of the cells. Error bars represent SEM (n = 1).

Ethylene emission from *Chlamydomonas* cells was monitored by laser photoacoustic ethylene detector. Despite the superior sensitivity of the equipment, ethylene production could not reliable be measured under flow- through conditions. Therefore, ethylene was allowed to accumulate for successive
periods of 100 min. Stimulation of ethylene production to average level of 0.06 nl/h was detected in response to treatment with 1 μM MP (Fig. 3A). To investigate the production of NO in MP-stimulated cells, advanced quantum laser-based spectrometry was employed. Basal production of NO (0.4 nl/h) was detected in control, non-treated cells. MP-treatment caused a release of up to 5.8 nl/h NO (Fig. 3B). The results revealed that in MP-treated Chlamydomonas cells ethylene and NO were produced and gas generation was associated with dramatic induction of cell death. This provides evidence that NO and ethylene participate in the mediation of MP-induced cell death. In response to MP-treatment higher levels of NO for 75 min. accumulation time, were detected (Fig. 3B) in comparison to ethylene released for 100 min. accumulation (Fig. 3A). This indicates a potential of the algal cells to generate large quantity of NO, but does not provide evidence for a dose-dependent priority of NO over ethylene in cell death signalling. Due to functional differences in the laser-based systems and the software used, timely synchronized simultaneous on-line detection of both gases was not performed, which in turn, makes difficult to estimate whether the augmentation of NO precedes the ethylene production or visa versa and, to distinguish a pivotal role of one of the volatiles.

In conclusion, we assume that cell death induced by MP is accompanied by stimulation of phospholipid signaling, including phospholipase C and D. Ethylene and NO are involved in the mediation of MP-stimulated cell death. The morphological features of the dead cells and the studied biochemical pathways in MP-treated Chlamydomonas cells show similarity to PCD in animal systems and to plant hypersensitive response.

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