Chemistry and Physics of Lipids 159 (2009) 13–20

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Cerium elicitor-induced phosphatidic acid triggers apoptotic signaling development in *Taxus cuspidata* cell suspension cultures

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ARTICLE INFO

Article history:
Received 19 October 2008
Received in revised form 25 January 2009
Accepted 5 February 2009
Available online 21 February 2009

Keywords:
Phosphatidic acid
Phosphaplipase D
LC–MS
Apoptosis
Signaling development
Taxus cuspidata cells

ABSTRACT

Degradation of membrane phospholipids is associated with apoptotic responses, but the signaling development of this degradation is not well understood. Cerium (Ce^{4+}), an important rare earth element, induces cellular apoptosis and taxol biosynthesis in *Taxus cuspidata* suspension cultures. Here, using mass spectrometry and biochemical technique, we demonstrated that the phospholipase D (PLD) was rapidly activated by Ce^{4+} and hydrolyzed structural phospholipids to generate lipid signal molecule, phosphatidic acid (PA). 1-Butanol, an antagonist of PLD-dependent PA production, blocked the biphasic burst of superoxide anions ($O_2^{\bullet -}$) and thus mitigated cellular apoptosis. The time-course analysis of PA accumulation and ERK-like mitogen-activated protein kinase (MAPK) regulation indicated PA generation preceded MAPK activation, suggesting that the rapid accumulation of PA might be required for the initial MAPK activity. After 2 h of Ce^{4+} elicitation, however, PA-induced $O_2^{\bullet -}$ burst, forming a negative regulation to MAPK activity, which in turn led to apoptotic signaling development.

1. Introduction

Hydrolysis of common membrane phospholipids occurs in different forms of programmed cell death (PCD) or apoptosis, and the cleavage products of phospholipids, such as phosphatidic acid (PA), are emerging as important second messengers in plants cell death development (Cristea and Esposti, 2004; Wang et al., 2006). The activities of several phospholipase including phospholipase A, phospholipase C (PLC), and phospholipase D (PLD) have been described during the apoptotic development, and the activation of PLD is believed to act as an important step not only in stress-induced phospholipid hydrolysis but also in cellular lipid signaling and apoptotic regulation (Testerink and Munnik, 2005; Nakashima and Nozawa, 1999). The burst of reactive oxygen species (ROS) is another striking event that occurs during the early phase of apoptosis (Lam, 2004; Zhao et al., 2005). In animals, a potential mechanism of PA action is the activation of the NADPH oxidase that produces ROS (Palicz et al., 2001). And also it was discovered that PA generation could effectively activate NADH oxidase for ROS production in tobacco, tomato and *Arabidopsis thaliana* plants, in a manner analogous to that found in mammal cells (de Jong et al., 2004; Laxalt et al., 2007; Andersson et al., 2006).

Recent evidences show that diverse extracellular stimuli, such as abiotic or biotic elicitors, are transduced into intracellular response by a mitogen-activated protein kinase (MAPK) cascade, and there exists a complex cross-talk mechanism between ROS burst and MAPK pathways in hypertensive response-like cell death of plants (Zhang and Klessig, 2001; Zhang et al., 2006). Moreover, inhibition experiments of PA generation suggest that PA may serve a key role in mediating MAPK activity in *A. thaliana* and *Triticum turgidum* cells (Lee et al., 2001; Zhang et al., 2003; Komis et al., 2006). Nevertheless, to date, how exactly these signal molecules regulated apoptotic signaling development in the elicitors-induced plants is not well known.

Plant cell culture has been developed as a promising alternative for the production of the secondary metabolite, taxol, an excellent anticancer drug (Yukimune et al., 1996). Due to the low yield of taxol in *Taxus cuspidata* cells cultures, many strategies, especial addition of elicitors, have been proposed to enhance taxol production (Yuan et al., 2001; Kim et al., 2006; Cheng and Yuan, 2006; Zhang and Fevereiro, 2007). Cerium is an important element of the rare earth elements, and its effect on development, differentiation and lesion in plants and animals has attracted much attention since the 1990s (He and Loh, 2000; Sun et al., 2003; Wu et al., 2005). Our previous studies indicated that cerium, especially Ce^{4+} ($Ce(NH_4)_2(NO_3)_6$), are very effective abiotic elicitors, bring the maximum taxol concentration to 5-fold of the control (Yuan et al., 1998, 2002a,b). Moreover, this abiotic elicitor induced not only the production and release of taxol, but also apoptosis (Yuan et al., 2002a,b). After discovering that the enhancement of taxol

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doi:10.1016/j.chemphyslip.2009.02.004

biosynthesis was accompanied by apoptosis in *T. cuspidata* cells, it becomes imperative to understand intercellular signaling involved in the Ce^{4+}-induced apoptotic response.

The production of superoxide anion ($O_2^{\bullet -}$) and the regulation of ERK (extracellular signal-regulated kinase)-like MAPK pathways have been found to play a signaling role during the apoptosis of Ce^{4+}-induced *T. cuspidata* cells, respectively (Yuan et al., 2002a,b; Ge et al., 2006). However, there is still insufficient knowledge of Ce^{4+}-induced apoptotic signaling development and taxol biosynthesis. In this work, we hypothesize a role for PA downstream of Ce^{4+} perception and were investigated (i) the activation of PLD and the resultant PA generation; (ii) the function of PA in reported $O_2^{\bullet -}$ burst; and (iii) the function of PA and $O_2^{\bullet -}$ in the regulation of ERK-like MAPK and apoptosis.

2. Materials and methods

2.1. Materials

Ammonium cerium nitrate ($Ce(NH_4)_2(NO_3)_6$) was purchased from Aldrich (Milwaukee, MI, USA). Cerium nitrate hexahydrate ($Ce(NO_3)_3{\cdot}6H_2O$), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (sodium salt) (14:0/14:0 PA), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0/14:0 PE), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0 PC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-l-serine sodium salt (16:0/16:0, PS), 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (sodium salt) (14:0/14:0 PG), l-α-phosphatidylinositol (PI) ammonium salt solution from bovine live were purchased from Sigma (St. Louis, MO, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphobutanol (sodium salt) and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (18:1-12:0 NBD-PC) were from Avanti Polar Lipids (Alabaster, AL, USA). Phosphospecific ERK (antip-ERK1/2, p44/42) antibody and specific ERK antibody (anti-ERK1/2, p44/42) were purchased from Santa Cruz Biotechnology. All other chemicals, with noted exceptions, were obtained from Sigma or Merck (Darmstadt, Germany).

2.2. Cell culture and elicitation

The cell lines were derived from young stems of *T. cuspidata* and subcultured onto solid B5 medium at 25 °C in the dark (Han and Yuan, 2004). Cell suspensions were cultured every 10 days for a total of five generations in freshly modified B5 medium containing sucrose (25 g L^{-1}), naphthylacetic acid (2 mg L^{-1}) and 6-benzyl aminopurine (0.15 mg L^{-1}). The suspensions were maintained in 250 mL flasks at 25 °C with continuous shaking (110 rpm) in the dark. The pH of the medium was adjusted to 5.8.

Ce^{4+} and Ce^{3+} were dissolved in distilled water to obtain a 0.5 M stock solution, respectively. One hundred μL of above stock solution was added to 50 mL cultures in the exponential growing stage (at the 10th day) to make a final concentration of 1 mM Ce^{4+} and 1 mM Ce^{3+}. An inhibitor of NADPH oxidase, diphenyliodonium (DPI), was dissolved in dimethyl sulfoxide (DMSO). 5 or 10 μM of DPI or 0.5% 1-butanol was applied to the culture system 2 h prior to the addition of Ce^{4+} elicitor. In all experiments, the final concentration of DMSO was approximately 0.1% (v/v).

2.3. Lipid extraction

Total lipid extraction of *T. cuspidata* cells was performed following the method of Welti et al. (2002). Briefly, fresh cells (approximately 0.6 g) were immediately placed in 3 mL isopropanol with 0.01% butylated hydroxytoluene (BHT) at 75 °C. Appropriate amounts of mixed internal standards were added during lipid extraction. The final concentration of each internal standard was around 1 μM. The tubes were incubated at 75 °C for 15 min, and 1.5 mL chloroform and 0.6 mL ultrapure water were added. The tubes were shaken for 1 h followed by removal of the extract. The cells were re-extracted with chloroform/methanol (2:1) containing 0.01% BHT five times with 30 min of agitation each time. The combined extracts were washed once with 1 mL 1 M KCl and once with 2 mL ultrapure water. The solvent was then evaporated under nitrogen, and the resulting lipid samples were stored at −20 °C. The remaining cells were heated overnight at 105 °C and weighed. Prior to analysis, the extracted lipid samples were redissolved in 2 mL chloroform/methanol (1:1, v/v).

2.4. Analysis of phospholipids by LC–MS and ESI-MS/MS

Analysis of phospholipids was performed on a Waters LC–MS system consisting of a 600E HPLC with an autosampler coupled to a Quattro Micro API triple–quadruple mass spectrometer (Micromass, Manchester, UK). The phospholipids were separated on a Lichrosphere Si60 column (125 mm × 4 mm, 5 μm, Merck) with a guard column made of the same packing material. The column temperature was kept at 25 °C. Mobile phase A was chloroform/methanol/ammonium hydroxide (30%) (89.5:10:0.5, v/v/v) and mobile phase B was chloroform/methanol/ammonium hydroxide (30%)/water (55:39:0.5:5.5, v/v/v/v). The following linear gradient was used: 5%–20% B for 7 min, 20% B for 3 min, 20%–30% B for 5 min, 30%–50% B for 30 min, 50%–5% B for 5 min, and 5% B for 5 min. The total elution time was 55 min at 1 mL min^{-1}. Injection volume was 10 μL. The eluent from the column was split and 0.2 mL min^{-1} flow was directed into the ion source of mass spectrometer. The Quattro Micro triple–quadruple mass spectrometer was operated in negative ESI ion mode, with a capillary voltage of 3.0 kV. The source temperature was set at 100 °C, and the voltage of cone, extractor and RF lens was 30, 3, and 0.1 V, respectively. Nitrogen was used as the desolvation gas and cone gas with flow rates set at 400 L h^{-1} (with a desolvation temperature of 350 °C) and 50 L h^{-1}, respectively. The Quattro mass spectrometer was operated in full scan mode, scanning from m/z 500–1000 with a scan time of 500 ms and an inter-scan delay time of 100 ms, resulting in approximately 100 scans per min. For phospholipid semi-quantification, the extracted ion chromatograms (EICs) were used. All peaks were integrated using the Masslynx Quanlynx Applications Manager (version 4.1) software. ApexTrack™ peak detection algorithm was applied after correction for the contribution from the ^{13}C isotope peak. The semi-quantification of phospholipid molecular species was based on comparisons of individual ion peak areas with that of a corresponding internal standard (i.e. 14:0/14:0 PG, 14:0/14:0 PE, 14:0/14:0 PC, 18:0/18:0 PI, 14:0/14:0 PA and 16:0/16:0 PS).

In order to achieve a better signal response for the identification of the phospholipids molecular species, the lipid extracts in 1:1 chloroform/methanol were infused into the ion source at the flow rate of 30 μL min^{-1}. An argon pressure of 3×10^{-3} mbar and a source temperature of 100 °C were used. The Quattro mass spectrometer settings were essentially the same as described above, except the collision energy was set to 20–45 V. The PC and PE classes of lipids were analyzed in the positive ion mode. The others were analyzed in the negative ion mode. PC profiles were generated by precursor ion scanning at m/z 184^+ with collision energy of 30 V. PE profiles were generated by neutral loss scanning for a loss of 141 Da with collision energy of 20 V. PS profiles were generated by neutral loss scanning for a loss of 87 Da with collision energy of 25 V. PG and PA profiles were generated by precursor ion scanning for m/z 153^- with collision energy of 35 V. PI profiles were generated by precursor ion scanning for m/z 241^- with collision energy of 45 V. The fatty acid compositions of individual phospholipid species were determined by daughter ion analysis as previously described by Yang et al. (2008).