Extracellular ATP is Involved in the Salicylic Acid-Induced Cell Death in Suspension-Cultured Tobacco Cells

Hanqing Feng, Dongdong Guan, Kun Sun, Yi Fang, Yao Zhao and Lingyun Jia

(College of Life Science, Northwest Normal University, 730070 Lanzhou, China)

Abstract: Extracellular ATP (eATP) can function as a signaling molecule to regulate a wide range of cellular processes. We investigated the regulatory role of eATP in the cell death induced by salicylic acid (SA) in suspension-cultured tobacco (Nicotiana tabacum L.) cells. Treatment of tobacco suspension cells with SA induced cell death. The same treatment lowered the levels of eATP, accompanied by a decrease of both the respiratory O2 uptake and intracellular ATP levels in tobacco suspension cells. Treatment with β,γ-methyleneadenosine 5'-triphosphate (AMP-PCP), which is the non-hydrolysable analogue of ATP and can exclude eATP from binding sites of eATP receptors, also induced cell death in tobacco cell cultures. Treatment with exogenous ATP partially alleviated the cell death induced by SA. These observations suggest that eATP is involved in the SA-induced cell death in tobacco cell cultures.

Key words: Cell death, Extracellular ATP, Salicylic acid.

Introduction

Salicylic acid (SA) is an important signaling molecule in plant cells (Gaffney et al., 1993). The level of SA in the plant increases with bacterial and viral infections, and the increase of SA is required to activate the expression of pathogenesis-related (PR) genes and defense responses of plants to pathogen infection (Gaffney et al., 1993; Delaney et al., 1995; Shah, 2003). Plants infected with biotrophic fungi or oomycetes also show SA accumulation, and the plants accumulating SA showed enhanced susceptibility to fungi and oomycetes (Glazebrook, 2005). Some abiotic stresses, such as drought, chilling, heavy metal toxicity, and heat, can also increase the SA contents in the plant, which is found to play important roles in enhancing the resistance to these stressed conditions (Yuan and Lin, 2008).

Adenosine 5'-triphosphate (ATP) has been regarded as an intracellular energy currency molecule for many years. However, ATP in extracellular milieu of animal cells has recently been found to function as a signaling compound regulating many cellular processes through interaction with membrane-associated receptor proteins. These processes include neurotransmission, immune responses, cell growth, and cell death (Khakh and Burnstock, 2009). ATP has also been reported to exist in extracellular spaces of plant cells. Many studies demonstrated that extracellular ATP (eATP) can mediate diverse physiological processes of plants, such as growth of root-hair, defense responses to pathogen infection, cell viability, and thigmotropism (Roux and Steinebrunner, 2007; Tanaka et al., 2010; Sun et al., 2012). Furthermore, eATP can stimulate the production of many intracellular signaling molecules, such as cytosolic free calcium (\([\text{Ca}^{2+}]_{\text{cyt}}\)), nitric oxide (NO), and reactive oxygen species (ROS) (Roux and Steinebrunner, 2007; Tanaka et al., 2010; Sun et al., 2012). Most recently Choi et al. (2014) identified a receptor for plant eATP from Arabidopsis.

Chemical treatment of cultured tobacco cells with SA induced the expression of \(\text{PR-1}\) gene and lowered the level of eATP. In addition, blocking the reduction of eATP suppressed the SA-induced expression of \(\text{PR-1}\) gene (Chivasa et al., 2009). A similar phenomenon was also observed in Arabidopsis cell cultures (Chivasa et al., 2009). These observations suggest that the SA-induced pathogen defense response could be mediated by eATP.

Besides inducing the defense (or resistance) responses to biotic or abiotic stresses, chemical treatment with SA has been found to cause cell death of plants, especially at high concentrations or under long-term application (Qiao et al., 2003; Broderse et al., 2005). A high concentration of SA, typically found in the pathogen-infected areas of the plant, is required for mounting the hypersensitive cell death, known as a defense response to restrict pathogen

Received 16 April 2014. Accepted 29 August 2014. Corresponding authors: H.Q. Feng (hanqing_feng@hotmail.com, fax +86-931-7971207).

Abbreviations: AMP-PCP, β,γ-methyleneadenosine 5'-triphosphate; iATP, intracellular ATP; eATP, extracellular ATP; FDA, fluorescein diacetate; ROS, reactive oxygen species; SA, salicylic acid.
growth and disease development (Alvarez, 2000; Gust and Nürnberg, 2012). Under cadmium stress, SA can accelerate the cell death of cadmium-stressed roots to avoid cadmium uptake by plants or to protect the stressed roots from cadmium-induced damage (Guo et al., 2009). Whether or not the SA-induced cell death is associated with eATP is not clear.

In the present study, we examined the effects of eATP on the SA-induced cell death. We believe that this research will further develop and expand the current knowledge about the physiological function of eATP and its role in SA-mediated physiological events.

**Material and Methods**

1. **Tobacco cell cultures**
   A tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell culture (cell line BY-2) was kindly provided by Prof. Jiang Liwen (The Chinese University of Hong Kong, Chian) and grown in suspension using Murashige and Skoog (MS) (Murashige and Skoog, 1962) liquid medium (pH 5.8) (Sigma-Aldrich) supplemented with 3% (w/v) sucrose and 0.4 mg L⁻¹ 2,4-dichlorophenoxy acetic acid under constant shaking at 25°C in darkness (Nagata et al., 1981). Cell suspension cultures were subcultured at 7-d growth cycle intervals by pipetting 10 mL of cell culture into 100 mL of fresh liquid medium. All procedures were done under aseptic conditions.

2. **Treatments of cells**
   All experiments were performed with the tobacco suspension cells at 3 d after subculture. In the first set of the experiments, the cell suspensions were subjected to 0, 100, 300, 500, or 700 μM SA, respectively, and incubated for 10 hr at 25°C in the darkness. In the second set of experiments, the cell suspensions were subjected to 0, 500, 1000 or 1500 μM AMP-PCP (β,γ-methyleneadenosine 5’-triphosphate), and incubated for 10 hr at 25 ± 1°C in darkness. In the third set of experiments, the cell suspensions were subjected to 50 μM ATP-Na₃, 500 μM SA, or 50 μM ATP-Na₃ plus 500 μM SA and then incubated for 10 hr at 25°C in the dark. The cell suspensions without any chemical treatment were used as the control.
3. Cell death assays

The cell suspensions were stained with fluorescein diacetate (FDA): living cells metabolize FDA to fluorogenic substrate fluorescein, so they emit green light after a excitation (Guilbault and Kramer, 1964). The cell suspensions were treated with 40 mg L\(^{-1}\) fluorescein diacetate (FDA, Sigma-Aldrich) and incubated in the dark for 10 min at room temperature. The samples were visualized under the fluorescent microscope (Leica, DM5000 B, Wetzlar, Germany).

Cell death was also quantified using the Evans blue staining assay as described by Yang et al. (2004) with some modifications. Cells with damaged membranes take up Evans blue dye, whereas viable cells that retain intact plasma membranes can exclude the dye (Kawai and Uchimiya, 2000; Hung et al., 2007). A 1-mL aliquot of the cell suspensions were stained for 8 min with 5 mL of 0.25% (w/v) Evans blue solution and then washed five times with phosphate buffered saline (PBS) to remove the excess and unbound dye. The dye bound to dead cells was solubilized in a solution containing 1% (w/v) SDS (sodium dodecyl sulphate) and 50% methanol (v/v) for 30 min at 50°C. The extracted dye was determined spectrophotometrically at 600 nm.

4. Measurements of intracellular ATP and extracellular ATP

The levels of intracellular ATP (iATP) and eATP of the cell suspensions were assayed using an ATP Bioluminescence Detection Kit (Beyotime Institute of Biotechnology, Haimen, China) by using luciferase to catalyze the release of light by ATP and luciferin (Stemler et al., 1987). For the measurement of iATP, the cell suspensions were centrifuged at 3500 × g for 4 min at room temperature, and the precipitate was washed with ATP-free liquid medium to remove the residual eATP. The intracellular milieu was extracted and ATP in the intracellular milieu was measured following the manufacturers instructions. For the measurement of eATP, the cell-free supernatant from the cell suspensions was collected by the method described by Mills and Lee (1996), and the measurement of ATP in the supernatant was performed as described by the manufacturer's instructions.

5. Respiratory O\(_2\) uptake

The cells in the culture medium were transferred to a 3-mL air-tight cuvette. The oxygen uptake of the cells was monitored using a Clark-type oxygen electrode (SP-2 type, constructed by the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China). The steady rate of respiratory O\(_2\) uptake was measured and calculated as described by Bingham and Farrar (1989).

Fig. 2. The effects of exogenous SA on the levels of eATP (A), iATP (B), and the respiratory O\(_2\) uptake (C) of tobacco cell-suspension culture. The levels of eATP and iATP are expressed as a percentage to the initial amount of ATP of the cell-suspension culture without SA treatment (0 \(\mu\)mol). Each value represents the mean ± SD of at least four independent experiments. The means denoted by the same letter did not significantly differ at \(P < 0.05\).
6. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The data were analysed using the Kruskall–Wallis one-way analysis of variance test. *P* < 0.05 was considered statistically significant.

**Results**

1. SA induced cell death in tobacco cell cultures

The tobacco cell cultures were treated with exogenous SA at 0 to 700 μM. FDA staining was used as a qualitative tool to visualize the SA-induce cell death. Microscopic observation showed that FDA fluorescence decreased with the increase in exogenous SA concentrations (Fig. 1A). The levels of cell death were also quantified by the Evans blue staining method. The results showed that the levels of cell death increased with the increase in the concentration of exogenous SA (Fig. 1B).

2. SA decreased the levels of eATP, iATP and the respiratory O_2 uptake of tobacco cell-suspension culture

Treatment with exogenous SA significantly decreased the levels of eATP. Treatment with 100, 300, 500, and 700 μM SA decreased eATP levels by 20.0, 37.2, 54.6 and 67.7%, respectively (Fig. 2A). Exogenous SA also decreased the iATP levels. The levels of iATP level were decreased by 5.9, 19.8, 27.7, and 32.8%, after the treatment with 100, 300, 500, and 700 μM SA, respectively (Fig. 2B).

As shown in Fig. 2C, treatment of the cell cultures with exogenous SA decreased respiratory O_2 uptake in a dose-dependent manner, which is similar to the change in the levels of eATP and iATP after the treatment with exogenous SA (Fig. 2A, B).

3. AMP-PCP induced cell death of tobacco cell cultures

In the present work, the tobacco suspension cells were treated with exogenous AMP-PCP (β,γ-methyleneadenosine 5-triphosphate). AMP-PCP is a non-hydrolysable analogue...
4. Addition of eATP alleviated the cell death induced by SA

To examine whether the induction of cell death by SA is linked to the depletion of eATP, we added exogenous ATP (50 μM) to the SA (500 μM)-treated tobacco cell cultures to limit the reduction of eATP due to the application of SA. Treatment with 50 μM exogenous ATP alone had no significant effect on the cell viability, compared to the control (cells without any chemical treatment) (Fig. 4). However, the cells subjected to the combined treatment with 500 μM SA plus 50 μM ATP had a lower cell death rate, compared to the cells treated with 500 μM SA alone (Fig. 4). These observations indicate that the SA-induced cell death could be associated with a decrease in eATP level.

Discussion

In the present work, treatment of tobacco suspension cell with SA induced cell death (Fig. 1). The induction of cell death by SA was accompanied by the decrease of both the respiratory O₂ uptake and iATP levels (Fig. 2B, C). SA...
is thought to impair the mitochondrial electron transport chain (mETC) and TCA (tricarboxylic acid) cycle (Norman et al., 2004; Rüffer et al., 1995), both of which are important for the respiratory oxygen reduction and the production of iATP (Mackenzie and McIntosh, 1999). In addition, the disruption of mETC and the decline of iATP production are found to play important roles in triggering cell death responses (Lam et al., 2001; Rhoads et al., 2006; Vidal et al., 2007). Thus, the observed cell death and the decrease of the respiratory O₂ uptake and iATP could be the result of impaired mitochondrial respiratory metabolism caused by SA.

Similar to the change of iATP level, SA significantly lowered the eATP levels (Fig. 2A). ATP synthase has not been found in the cell surface or plasma membrane of plant cells. Previous studies have revealed that plant cells can release ATP from the iATP pool either via the ATP-binding cassette transporters or exocytosis (Kim et al., 2006; Thomas et al., 2000). Thus, eATP originates from iATP that is released into the extracellular milieu (Khakh and Burnstock, 2009; Tanaka et al., 2010). This means that any change in iATP level would have an important influence on the level of eATP. Mitochondrial ATP is the major resource of iATP. Thus, it is reasonable to assume that SA decreased the mitochondrial ATP production by impairing mitochondrial respiratory metabolism and subsequently led to the decline of eATP levels.

In the last few decades, the SA-mediated physiological events responsible for the induction of cell death have been described in the intracellular milieu (Alvarez, 2000; Kawai-Yamada et al., 2004; Gust and Nürnberg, 2012). In the present work, we further investigated whether the decline of eATP level in the presence of SA, as an extracellular event, is involved in the SA-induced plant cell death. Because of its high charge, eATP cannot passively diffuse across the plasma membrane. eATP binds to the site of putative eATP receptors/ATP-binding proteins located in the plasma membrane, where hydrolysis of ATP occurs. The hydrolysis of ATP, following binding, is crucial in triggering signal transduction and physiological events (Song et al., 2006; Chivasa et al., 2009, 2010). In the present work, the effect of eATP on the viability of tobacco suspension cells were studied by using AMP-PCP (βγ-methyleneadenosine 5-triphosphate). As mentioned above, AMP-PCP has antagonistic effects on the physiological processes requiring eATP. Exogenous application of AMP-PCP induced cell death of tobacco cell cultures (Fig. 3), suggesting that the decrease in eATP level could initiate cell death. Furthermore, the treatment with exogenous ATP partially alleviated the cell death induced by SA (Fig. 4). These observations suggest that SA induces cell death through a signaling pathway that is associated with a decline of eATP level.

The mechanism of the alleviative effect of eATP on the SA-induced cell death is not clear. In animal cells, eATP can stimulate the accumulation of [Ca²⁺]cyt by binding and activating the membrane-associated P2-type purinoceptor protein (Dichmann et al., 2000; Abbracchio et al. 2006). Treatment with exogenous ATP can also result in the specific accumulation of [Ca²⁺]cyt in plant cells (Demidchik et al. 2009). Although genomic sequence–based surveys to identify plant eATP receptors homology to animal purinoceptors failed to find any suitable candidate proteins, Choi et al. (2014) revealed that the DORN1 protein of Arabidopsis binds eATP with high affinity and is required for eATP-induced accumulation of [Ca²⁺]cyt.

Previous studies revealed that an increase in Ca²⁺ level can enhance the production of iATP by increasing the availability of mitochondrial substrates (Jouaville et al. 1999: Logan and Knight 2003). Thus, if, as suggested above, the SA-induced cell death is a result of a decline of iATP production due to the inhibition of the mitochondrial respiration by SA, it is reasonable to assume that the increase in Ca²⁺ level by eATP could alleviate the SA-induced inhibition in respiration and iATP production, and thus rescue tobacco suspension cells from SA-induced death. However, future work will be needed to confirm the mechanism.

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