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Antioxidative cellular response of lepidopteran ovarian cells to photoactivated alpha-terthienyl

Qingchun Huang [a,*], Xinming Yun [a], Wenbing Rao [a], Ciying Xiao [b]

[a] Shanghai Key Lab of Chemical Biology, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, PR China
[b] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, PR China

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ABSTRACT

Photodynamic sensitizers as useful alternative agents have been used for population control against insect pests, and the response of insect ovarian cells towards the photosensitizers is gaining attention because of the next reproduction. In this paper, antioxidative responses of lepidopteran ovarian Tn5B1-4 and Sf-21 cells to photoactivated alpha-terthienyl (PAT) are investigated. PAT shows positive inhibitory cytotoxicity on the two ovarian cells, and its inhibition on cell viability is enhanced as the concentrations are increased and the irradiation time is extended. Median inhibitory concentrations (IC_{50}) are 3.36 μg/ml to Tn5B1-4 cells, and 3.15 μg/ml to Sf-21 cells at 15 min-UV-A irradiation 2 h-dark incubation. Under 10.0 μg/ml PAT exposure, 15 min-UV-A irradiation excites higher ROS production than 5 min-UV-A irradiation does in the ovarian cells, the maximum ROS content is about 7.1 times in Tn5B1-4 cells and 4.3 times in Sf-21 cells, and the maximum malondialdehyde levels in Tn5B1-4 and Sf-21 cells are about 1.47- and 1.36-fold higher than the control groups, respectively. Oxidative stress generated by PAT strongly decreases the activities of POD, SOD and CAT, and induces an accumulation of Tn5B1-4 cells in S phase and Sf-21 cells in G2/M phase in a concentration-dependent fashion. Apoptosis accumulation of Tn5B1-4 cells and the persistent post-irradiation cytotoxicity are further observed, indicating different antioxidative tolerance and arrest pattern of the two ovarian cells towards the cytotoxicity of PAT.

1. Introduction

Photoactivated degradation has always been an inherent weakness for most traditional organophosphate and carbamate insecticides in guaranteeing continued bumper harvests in agriculture. In recent years, the photodynamic sensitizers such as thiophenes, xanthenes, acridines, porphyrins, furanocoumarins, phenothiazines, and polyacetylenes, however, have been successively reported as photoinsecticides which become efficacious alternative agents to control the insect pests through the activation of light [1,2].

These photoinsecticides have the advantages of high selectivity on pests. For example, phloxine B (2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachloro-fluorescein) that showed excellent insecticidal activity in controlling cucumber beetles, corn rootworms and fruit flies, owns 100 thousand times higher safety on human than malathion [3,4]. When being exposed to light, the photoinsecticides undergo rapid activation on the formation of singlet oxygen and related reactive oxygen species (ROS), which can destroy many biological components such as lipids, proteins, or DNA bases in cells and insect tissue, causing great improvement on their effect in killing insects [5,6]. These photoinsecticides act on multiple targets accompanying with lower resistant risk against insect pests and no cross-resistance with traditional insecticides [7]. After the activation, the photoinsecticides are transformed into non-phototoxic and pollution-free residues in the environment.

Alpha-terthienyl, a secondary metabolite extracted from the root of the marigold, is a natural phototoxin with strong ability in generating singlet oxygen ($^1O_2$) and lipid peroxidation [8]. It has been developed as an effective photoactivated insecticide against pests by acting on the neuromuscular sheath, the midgut membranes, the cuticle enzymes, even inducing the development of insect into abnormal pupaem [9–12]. Many terthienyl derivatives have been developed such as cyano-alpha-terthienyls, thienyl 1,3,4-thia(oxa)diazoles, alpha-terthienyl-conjugated pyrethroids with an enhanced efficacy in reducing the populations of mosquitoes and other pests [13–15]. These developments of terthienyl derivatives increase the expanded demand for clear understanding of the detail photoactive mechanism, and the response of insect ovarian cells towards the photosensitizers is gaining attention. Hence, the effect of photoactivated alpha-terthienyl (PAT) on lepidopteran ovarian Tn5B1-4 and Sf-21 cells is investigated by comparing the different antioxidative response of ovarian cells on the cytotoxicity

* Corresponding author at: Shanghai Key Lab of Chemical Biology, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China.
E-mail address: qchuang@ecust.edu.cn (Q. Huang).

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of PAT through monitoring cell viability, the induction of intracellular ROS, the increase of malondialdehyde (MDA) level, the generation of oxidative stress, and the modulation of cell cycle and apoptosis. The aim is to provide experimental evidences at the cell level for the interference of population control using photosensitizers in agricultural practice.

2. Materials and methods

2.1. Chemicals

Alpha-terthienyl, trypan blue, nitroblue tetrazolium (NBT), and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich Chemicals (Bornem, Belgium). Fetal bovine serum (FBS) was from PAA Laboratories. TNM-FH medium were from HyClone. Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), n-butanol, propidium iodide (PI), phenylmethylsulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and other chemicals were purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China).

Alpha-terthienyl was dissolved in acetone to prepare a stock solution with a concentration of 1000 μg/ml, and placed at 4 °C away from light. The solution was then diluted in culture media to desired concentrations for experimental use.

2.2. Cell cultures

The *Trichoplusia ni* ovarian Tn5B1-4 cell line and *Spodoptera frugiperda* ovarian Sf-21 cell line, that were purchased from Cell Bank (Chinese Academy of Sciences, Shanghai, China), were cultured at 27 °C in TNM-FH medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 mg/ml streptomycin. Cells were collected after gentle pipetting, and washed twice in 0.1 M cold PBS buffer (8.00 g NaCl, 0.20 g KCl, 1.29 g $Na_2HPO_4 \cdot 3H_2O$, 0.20 g KH_2PO_4, 1000 ml ddH_2O, pH 7.4), followed by incubation in 96-well plates with fresh medium containing alpha-terthienyl.

2.3. Photoactivated exposure

Cells at exponentially growing stage were seeded in 96-well plate and incubated in TNM-FH medium until 70% cell confluence was attained. After the medium were removed, the cells were washed twice with cold PBS buffer, and cultured continuously in fresh medium containing 1, 2, 5, 7, or 10 μg/ml alpha-terthienyl with 0.1% acetone, with alpha-terthienyl-free medium as a control. The cultures were then irradiated to achieve maximum intensity under a UV-A light (40 W, UV 250 mW cm^{-2}, wavelength 320–400 nm, 17 cm distance) at room temperature. Identical cultures that were placed in dark for the same time were used as positive control. After UV-A irradiation, all cultures were allowed to incubate in dark for 2 h, 4 h, or 24 h. All treatments were used for next determination.

2.4. Trypan blue dye exclusion assay

For evaluating the effect of PAT in inducing cytotoxicity, cells (1.0×10^5 cell/ml) in 5, 10, 15, 20, or 30 min-UV-A irradiation 2 h-dark incubation were re-suspended in 200 μl fresh medium, and cell viability was assessed using trypan blue dye exclusion assay according to Gutting et al. [16] with minor modification [16]. After the treated cells were incubated with 0.4% trypan blue for 5 min, cells were loaded into a hematocytometer and the number of the cells with different dye uptake was counted. The clear cells that maintained cell membrane integrity and did not take up the dye were lived, and the blue cells with damaged membrane and the accumulation of dye were counted as dead. PAT inhibition values, in triplicate, were expressed as a percentage of the total cell population by counting viable and dead cells.

2.5. Detection of intracellular ROS

The content of intracellular ROS was measured using DCF-DA as previous reported method [12]. Briefly, cells (1.0×10^5 cell/ml) in 5, or 15 min-UV-A irradiation 2 h-dark incubation were loaded with 5 μg/ml of DCF-DA in the medium and incubated for 2 h. The excess of DCF-DA was removed by washing with PBS. ROS generation of cells was examined by using a Fluorescent microtiter plate photometer (Synergy2 BioTek) at 485 nm for excitation and 530 nm for emission. The results represented the percentage of ROS production in PAT treatments relative to that in the negative control which was treated only with fresh medium and acetone 0.1%.

2.6. Measurement of MDA levels

The level of MDA was determined in order to measure lipid peroxidation according to the method described by Stocks et al. [17]. The cells (2.0×10^5 cell/ml) in 15 min-UV-A irradiation 2 h-dark incubation were collected and homogenized with 1.0 ml of 10% (w/v) TCA, and then centrifuged (4000 ×*g*, 4 °C) for 10 min. The supernatant (1.0 ml) was mixed with 1.0 ml of 0.6% thiobarbituric acid (in 10% TCA) and heated in boiling water for 15 min. After cooling, 0.5 ml of n-butanol was added to the mixture with sufficient mixing, and then centrifuged at 4000 ×*g* in room temperature for 10 min. The absorbance of the supernatant was measured at 532 nm. The MDA level was calculated by the absorbance coefficient of MDA-TBA complex, 1.56×10^5 l/mol·cm and expressed in nanomoles per liter.

2.7. Enzymatic measurements

The cells (2.0×10^5 cell/ml) in 15 min-UV-A irradiation 2 h-dark incubation were collected and washed twice with PBS, and then lysed in 50 mM pre-cold Tris-HCl buffer (containing 150 mM NaCl, 1.0 mM PMSF, 1.0 mM EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, pH 7.4) for 15 min. The lysates were centrifuged (10,000 ×*g*, 4 °C) for 10 min, and then supernatants were collected. The supernatant after added in 0.1 M pre-cold sodium acetate buffer (pH 5.0) was assayed for peroxidase (POD) activity, and the supernatant after added in 0.05 M pre-cold phosphate buffer (containing 1.0 mM polyvinyl pyrrolidone, pH 7.0) was assayed for the activity of superoxide dismutase (SOD) and catalase (CAT).

POD activity was measured according to Simon et al. [18] at a wavelength of 470 nm using guaiacol as substrate [18]. SOD activity was measured using the reaction system contained 13.0 mM D-methionine, 75.0 μM NBT, 100.0 nM EDTA, and 6.0 μM riboflavin with colorimetry at 560 nm as described by Beauchamp et al. [19]. CAT activity was determined kinetically through the method described by Aebi [20] based on the decomposition of H_2O_2 measuring the decrease in absorbance at 240 nm [20]. The results were normalized to the protein content that was determined at 595 nm using the Bradford method [21].

2.8. Analysis of cell cycle and apoptosis

Cell cycle and apoptosis analysis was performed with a flow cytometry as described by Gougeon et al. [22]. The cells (1.0×10^6 cells/ml) in 15 min-UV-A irradiation 4 h- or 24 h-dark incubation were harvested, suspended in 0.6 ml of cold PBS, fixed and permeabilized by adding 1.4 ml of 70% ethanol at −20 °C overnight. The cells were centrifuged (1000 ×*g*, 4 °C) for 5 min, lysed in 1.0 ml of 50 μg/ml RNaseA for 30 min, incubated with 1.0 ml of 50 μg/ml PI in dark at room temperature for 30 min, and then analyzed with a flow cytometer (FACSCalibur, Becton Dickinson, NJ) and CellQuest software procedure. Plots were

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