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Pyrethrum-extract induced autophagy in insect cells: A new target?

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ABSTRACT

Pyrethrum extract (PY) is a natural insecticide that is extensively used across the world, and its insecticidal activity is attributed to the presence of six active esters known as pyrethrins. PY targets the nervous systems of insects by delaying the closure of voltage-gated sodium ion channels in the nerve cells. However, limited information is available regarding the toxicity and detailed mechanisms of PY activity. This study is aimed at understanding the toxicity effect and the underlying mechanisms of PY in cellular level, which have not yet been investigated on the non-nervous system of insects. Results of the MTT assay showed that the viability of Sf9 cells was inhibited by PY in a time- and concentration-dependent manner, and observation under a microscope revealed accumulation of intracellular vacuoles. Monodansylcadaverine staining analysis and transmission electron microscope images revealed typical autophagic morphological changes in PY-treated Sf9 cells. Autophagy-related proteins such as LC3, p62, and beclin-1 were detected using by Western blotting. Protein expression levels of LC3-II and beclin-1 were upregulated while that of p62 was markedly downregulated in a dose-dependent manner upon the PY treatment in Sf9 cells. In conclusion, these results indicate that PY could induce autophagy in the non-nervous system of insects which may contribute to its insecticidal mechanism.

1. Introduction

Pyrethrum extract (PY), a natural insecticide derived from the flowers of *Chrysanthemum cinerariaefolium*, is widely used for household and other applications [1]. The insecticidal activity of PY is attributed to the presence of six active esters known as pyrethrins, which comprise two groups, namely pyrethrin-I (pyrethrin I, cinerin I, and jasmolin I) and pyrethrin-II (pyrethrin II, cinerin II, and jasmolin II) [2]. PY mainly exerts direct actions on the nervous systems of insects. Due to its high efficiency and low toxicity to mammals, it has been successfully used to control pest in organic farming and manage residential indoor pest [3,4]. Till date, many studies have investigated the neurotoxicity effects of PY in insects [5,6], and other studies have also reported the toxicity effects of PY in nontarget organisms such as aquatic life and the liver of rats [7,8], although the definite mechanism is still poorly understood [9].

Autophagy is an important cellular catabolic process in which portions of the cytoplasm are sequestered into double-membrane vesicles known as autophagosomes, which represent the morphological characteristics of autophagy [10]. This autophagosome then fuses with the lysosome where the contents are degraded and recycled. In recent years, increasing evidence has demonstrated that autophagy can cooperate with or support alternative routes of cell death and that persistent stress can promote autophagic cell death, which is distinct from other forms of cell death [11]. This is also known as programmed type II cell death [12,13]. Briefly, autophagy not only displays cell morphological characteristics represented by autophagosomes but also drives and controls a group of autophagy-related proteins (ATGs), which are highly conserved from yeast to humans [14], such as the protein light chain 3 (LC3, also known as ATG8), p62/SQSTM1 ubiquitin-binding protein, which is also known as "autophagic flux", and beclin-1 (or ATG5). These activities strongly indicate the existence of various forms of autophagy and the dynamics of the autophagic process.

The *Spodoptera frugiperda* (Sf9) cell line, as a typical insect non-nerve cells, was used to characterize the insecticidal mechanism of PY in cellular level. In this study, we used various bioassays to reveal the cytotoxic effects of PY on Sf9 cells, including the MTT assay, cellular morphology observation assay, autophagic vacuoles assay, transmission electron microscopes analysis and Western blotting assay. The results showed that the viability of Sf9 cells was inhibited and intracellular vacuoles were accumulated. Moreover, changes of typical autophagic morphological were carried out. And the expression levels of LC3-II and beclin-1 were upregulated while that of p62 was markedly downregulated in a dose-dependent manner upon the PY treatment in Sf9 cells. It demonstrated that PY could induce autophagy in Sf9 cells which may contribute to its insecticidal mechanism.

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2. Materials and methods

2.1. Reagents and chemicals

Pyrethrum-extract, 3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were bought from Sigma-Aldrich (St. Louis, MO, USA). The BCA Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). And the caspase-3 Activity Assay Kit was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was from Sangon Biotech Co., Ltd. (Shanghai, China). The other antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents and chemicals were of analytical grade and purchased from locally.

2.2. Cell culture

Spodoptera frugiperda (Sf9) cells obtained from American Type Culture Collection (ATCC) were cultured in Serum-Free insect Cell Culture medium (SFM) (Hyclone, Logan, Utah, USA). The media were supplemented with 10% (V/V) heatinactivated FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Hyclone, Logan, Utah, USA) and 100 μg/mL streptomycin (Hyclone, Logan, Utah, USA) at 28 °C in biochemical incubator.

2.3. . Cell viability assay

The effect of PY on the viability of Sf9 cells was measured separately by MTT assays [15]. At first, cells were seeded in to 96-well culture plates (1×10^4 cells per well). After incubation overnight, cells were incubated in fresh medium containing 5, 10, 20, 40 and 80 μg/mL PY with 0.1% dimethyl sulfoxide (DMSO) which was used as a negative control. Cells were treated with indicated concentrations of pyrethrum-extract for 24 and 48 h. Then, 20 μL of the prepared MTT (5 mg/mL) solution was added to each well and cells were incubated for 4 h until a purple precipitate was visible. The formed formazan crystals were dissolved in DMSO (150 μL/well) by constant shaking for 10 min. Absorbance was measured on a Synergy H1 microplate reader (Bio-Teck, Winooski, VT, USA) at a test wavelength of 492 nm and a reference wavelength of 630 nm. Each experiment was repeated at least three times in quadruplicate. Percentage of cell viability was calculated by the following formula: % cell viability rate = $[(\text{mean}(OD_{492} - OD_{630})\text{ in test wells}) / (\text{mean absorbency in control wells})] \times 100\%$.

2.4. Cellular morphology changes observation on Sf9 cells

Cellular morphology changes of PY-treaded Sf9 cells were observed by microscope (Lecois, DM3000). Cells were plated in 24-well plate at the density of 4×10^4. After incubating for 6 h with the 0, 5, 10, 20, 40 and 80 μg/mL of PY, the cell cultures were microscopically examined and photographed at all exposure levels.

2.5. Acidic vesicular organelles labeled by monodansylcadaverine (MDC)

Monodansylcadaverine (MDC) selectively labels autophagic vacuoles as described previously [16]. Briefly, Sf9 cells (1×10^4) were seeded into 6-well plates and cultured overnight, after treated with various concentrations (0, 5, 10, 20, 40 and 80 μg/mL) of PY for 6 h, cells were harvested and washed twice with PBS (pH 7.4). Subsequently the cells were incubated with MDC (20 μM) for 15 min in the dark. After incubation, cells were washed three times with PBS (pH 7.4). Then immediately analyzed by fluorescence microscope (Lecois, DM3000). The fluorescence intensity was measured using *ImageJ* software.

2.6. Transmission electron microscopes (TEM) analysis

To morphologically observe the induction of autophagy in PY-treated Sf9 cells, we performed electron microscopy analysis as described elsewhere [17]. Ultrastructural structure of PY-treated Sf9 cells was analyzed by transmission electron microscopes (TEM). Briefly, after being treated with the 0, 5, 10, 20, 40 and 80 μg/mL of PY for 6 h in 6-well plate, Sf9 cells were harvested and washed twice with PBS, then fixed with ice-cold glutaraldehyde (2.5% in 0.1 M PBS buffer, pH 7.4) for 30 min at 4 °C. The cell pellet were post-fixed in 1% phosphate-buffered osmium tetroxide (OsO_4) in the same buffer for 1 h at 4 °C and embedded in LX 122 before being cut and stained with uranyl acetate/lead citrate. Representative areas were chosen for ultrathin sectioning and were observed using a FEI Tecnai™ transmission electron microscope (TEM) (MODEL#: M3000).

2.7. Western blot analysis

Analysis of specific proteins was performed using western blot. After incubating by PY as described above, with 0.1% DMSO with PY-free treatment as a negative control, 2×10^6 cells were collected and centrifuged at 1000*g* for 5 min, washed three times with PBS (pH 7.4). Total proteins were extracted by the modified RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO, USA) for 30 min on ice and centrifuged at 12,000*g* for 5 min. The supernatant was collected and the protein contents of cell lysate were determined according to using the BCA Protein Assay kit. Equivalent quantities of proteins were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene-difluoride (PVDF) membrane (Millipore Corp., Atlanta, GA, USA), The transferred blot membranes were blocked in 5% non-fat dry milk dissolved in Tris-buffered saline-Tween (TBST) (10 mM Tris·HCl, pH 7.5; 150 mM NaCl; 0.1% Tween-20) for 2 h at room temperature, and probed with primary antibodies (diluted 1:1000 in TBST with 5% non-fat dry milk) overnight at 4 °C. Meanwhile, β-actin (diluted 1:1000) was using as loading control to normalize for protein content. After washing three times with TBST, the membranes were incubated in the appropriate Horse radish-peroxidase (HRP) conjugated secondary antibodies (diluted 1:5000 in TBST containing 5% non-fat dry milk) at room temperature for 2 h. Blots were revealed by ECL Western blot detection reagents. Visualization of the immunolabeled bands was carried out by autoradiography. All protein bands were scanned and integrated density values (IDVs) were quantified by *ImageJ* software.

2.8. Statistical analysis

Data are presented as the mean of at least three independent experiments along with standard deviation (SD). Differences were considered to be significant when P-values < 0.05. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by a t-test. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of PY on viability of Sf9 cells

The effect of PY on viability of Sf9 cells was determined by MTT assay. Sf9 cells were treated with PY (0–80 μg/mL) for 24 h and 48 h, and the results of cell viability assay in each treatment were displayed separately in Fig. 1. The results showed that PPY inhibited the viability of Sf9 cells in a both concentration- and time-dependent manner. PY at a concentration of 5–80 μg/mL reduced the cell viability by 22.23 ± 2.88, 37.13 ± 2.71, 55.64 ± 1.32, 59.26 ± 7.26 and 63.75 ± 1.82% at 24 h and 44.86 ± 2.51, 70.11 ± 1.57, 84.67 ± 0.98, 89.08 ± 0.91, and