Pesticide Biochemistry and Physiology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Attribution of Bax and mitochondrial permeability transition pore on cantharidin-induced apoptosis of Sf9 cells

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ARTICLE INFO

Article history:
Received 20 July 2016
Received in revised form 11 January 2017
Accepted 14 January 2017
Available online xxxx

Keywords:
Cantharidin
Sf9 cells
Bax channel
Mitochondrial permeability transition pore
Cytochrome C
Mitochondrial transmembrane permeability

ABSTRACT

To investigate the insecticidal mechanism of cantharidin, a promising biological pesticide substance from blister beetle, on Sf9 cells, a cultured cell line derived from fall armyworm, *Spodoptera frugiperda*, we preliminary studied the attribution of Bax channel and mitochondrial permeability transition pore on cantharidin-induced mitochondrial apoptosis signal pathway. Changes in cell morphology, activity of mitochondrial dehydrogenases, release of cytochrome C and mitochondrial transmembrane potential were detected when the two channels were blocked by specific inhibitors, Bax channel blocker and cyclosporin A. Results showed that cantharidin-induced apoptotic features, including changes in the cell morphology, release of cytochrome C and decrease in mitochondrial transmembrane potential could be significantly inhibited by Bax channel blocker, while cyclosporin A accelerated the downward trend of mitochondrial dehydrogenases activity and caused a decrease of Ca^{2+} in mitochondria. In summary, Bax might be necessary but not exclusively for the apoptosis induced by cantharidin and the attribution of these channels seems to be more complexity.

1. Introduction

Pests and pesticides has consistently been a hotspot in agriculture. Because of the excessively use of chemical pesticides, many pests have developed their resistance to lots of pesticides. Research showed that pests of Noctuidae have developed resistance to pyrethroid, organophosphate, carbamate, ecdysone analogs and many other commonly used pesticides [1,2]. Much more attentions have been paid to the environment friendly bio-pesticides, no matter botanical, animal-origin or microbe-produced pesticides.

Cantharidin ($C_{10}H_{12}0_4$, CTD), a sesquiterpenoid bioactive substance derived from Spanish fly or blister beetle (Coleoptera: Meloidae), including *Mylabris cichorii* L. and *Mylabris phalerata* Pallas, is used as a traditional Chinese medicine for thousands of years and it has many benefits on antitumor, urination and cleaning died tissues [3,4]. Discovery of its insecticidal activities, contact poisoning or antifeeding effect, were even made earlier than the advent of chemical pesticides [5,6]. And later its antifungal [7,8] and herbicidal effects came into view [9]. CTD has been registered as a new type of bio-pesticide in China. CTD aqueous solution (0.1%) showed high insecticide activity and long efficacy duration on controlling aphid and white butterfly on cabbage [10]. It is an efficient, safe, excellent new pesticide product [11–13], but the insecticidal mechanism of CTD remains a mystery.

Researches showed that CTD could cause potent contact poisoning, stomach action and anti-feeding effect in armyworm, *Leucania separata* Walker, and diamondback moth, *Plutella xylostella* L. [14]. Pathological changes noted in the midguts were much more like apoptosis characters in mammalian cells [15]. Further studies showed that CTD could induce apoptosis among Lepidopteran insect cell lines. Chromatin pyknosis, apoptotic body formation [16] and DNA ladders [17] all been observed. Ser/Thr protein phosphatases, especially protein phosphatase type 2A (PP2A), were considered as the target of CTD in medicine researches [18–20] and CTD could inhibit protein phosphatases of cotton bollworm, *Heliothis armigera* Hübner, and the oriental armyworm, *Mythimna separata* Walker, significantly [21,22] *in vivo* and *in vitro*.

Our laboratory had cloned the Sf-PP2Ac full-length DNA sequence of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and got its inclusion bodies [23,24]. But we know nothing about how apoptosis occur, except CTD could pass through the cell membrane easily and cause metabolic disorder and destruction of mitochondria [15,25,26].

Apoptosis is an important biological process both in mammals and insects. Researchers found that apoptosis in Lepidoptera was very similar to mammalian apoptosis and release of cytochrome C (Cyt C) was considered as the only thing necessary for the apoptosis [27,28]. In the case of ATP, released Cyt C in cytoplasm would activate the apoptotic protease activating facter-1 (Apaf-1) and its caspase recruitment domain to promote the formation of apoptotic bodies, which composed of Cyt C, Apaf-1 and procaspase 9. Then activated caspase 9, would lead to cascade reaction of apoptosis effector caspases, caspase 3, caspase 7, and so on [29–31].

Abbreviations: CTD, cantharidin; PP2A, protein phosphatase type 2A; C, cytochrome C; Apaf-1, apoptotic protease activating facter-1; MPTP, mitochondrial membrane permeability transition pore; MMP, mitochondrial membrane potential; CsA, cyclosporin A; BCB, Bax channel blocker; MDH, mitochondrial dehydrogenases; RFI, relative fluorescent intensity; MAPKs, mitogen-activated protein kinases.

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http://dx.doi.org/10.1016/j.pestbp.2017.01.010
0048-3575/

As to the special mechanisms of Cyt C release, mitochondrial membrane permeability transition pore (MPTP) and Bcl-2 family were mainly involved in the regulation of mitochondrial membrane permeability [32–34]. Openness of MPTP could make the mitochondria swell and the outer membrane rupture, leading to the release of Cyt C. As to Bax, the first discovered pro-apoptotic protein of Bcl-2 family, it would combine with another Bax or Bak and then insert into the mitochondrial membrane to form channels. Some others believed that Bax or Bak could regulate the change of mitochondrial membrane potential (MMP) [35], which could lead to pro-apoptotic factors release from the mitochondria membrane space [36–38]. Thus, these two types of channels, Bax channels and MPTP, played important roles in the transmitting of apoptosis signals to mitochondria.

In this article, special channel inhibitors, cyclosporin A (CsA) and Bax channel blocker (BCB) were used to explore the role of MPTP and Bax on CTD induced apoptosis in Sf9 cells. Morphological observation, enzyme activity assay, Western blot and fluorescence analysis were all used to detect the changes of apoptosis when those two channels were blocked. It would help to know the apoptotic signals transduction and the insecticidal mechanism of cantharidin.

2. Materials and methods

2.1. Cell lines and culture

Sf9 cells were obtained from Institute of Zoology, Chinese Academy of Science, and routinely maintained in cell culture flasks (Corning, USA) with Sf-900II SFM culture medium, containing 10% fetal bovine serum, 1% streptomycin and penicillin (Gibco, USA), in a constant temperature cell culture incubator (Thermo Electron Corporation, USA) under 27 °C. Cells were subcultured every 3 days, as the cell covered approximately 80% of the flask bottom. Cell numbers were determined with a hemocytometer and cell viability was determined using trypan blue exclusion test.

2.2. Observation of cell morphology

Sf9 cells were incubated for 48 h (approximately 10^6/mL) and then inoculated in 6-well plates with 2 mL/well for another 24 h. All reagents were dissolved in DMSO (AMRESCO, USA). After pretreatment with CsA (20 μM) and BCB (20 μM) (Tocris, UK) for 3 h, CTD (32 μM) (Sigma, USA) was added to each well. There were 6 experiment groups, namely normal cell group (CK), DMSO control group (DMSO), CTD treatment group (CTD), CTD treated after CsA's pretreatment (CsA), CTD treated after BCB's pretreatment (BCB) and CTD treated after CsA's and BCB's co-pretreatment (CsA-BCB). DMSO contents were same in each group except CK. Morphological changes were examined under inverted fluorescence microscope (Zeiss AX10, Germany) and microscopic imaging system (CRi Nuance, USA) at 30, 60, 90 and 120 min, 3 fields was randomly selected of each group.

2.3. Detection of the activity of mitochondrial dehydrogenases

Sf9 cell suspensions (1–5 × 10^5/mL) were incubated in 96-well plates (100 μL/well). CsA (20 μM) and BCB (20 μM) pretreated the cells 3 h before CTD treatment. DMSO was kept the same in 5 groups besides CK. After 60, 120 and 180 min, supernatants were replaced with culture mediums containing 0.5 mg/mL MTT (Sigma, USA). After additional 4 h incubation, the mediums were replaced by 150 μL DMSO to dissolve the formazan crystal. Optical densities were measured at 630 nm by a microplate spectrophotometer (Bio-Rad Model 680, USA) and 5 replicates per treatment.

2.4. Release of cytochrome C

Cells were cultured in 75 cm^2 cell culture flasks (Corning, USA) with approximately 10^7 cells/mL in 10 mL culture medium. After pretreating with CsA or BCB for 3 h, cells were treated with CTD (32 μM) for 1 h. There were about 1.20‰ DMSO in treatment groups except CK group. Carefully collected the cells (3000 g, 5 min, and 4 °C), washed with phosphate buffer saline twice and stored at −80 °C.

Mitochondrial and cytosolic fractions were separated by the Cell Mitochondria Isolation Kit (Beyotime, China). In brief, Sf9 cells were suspended in 200 μL mitochondria extraction buffer and incubated 10–15 min on the ice. Cells were homogenized and centrifuged (600*g*, 10 min) at 4 °C. Then the supernatants were spun at 12,000*g* for 10 min. Supernatants and pellets were used to prepare the cytosolic fraction and the mitochondrial fraction, respectively. The supernatants were centrifuged at 12,000*g* for another 10 min to remove the residue and saved as the cytosolic fraction. The pellet was suspended in 50 μL mitochondria lysis buffer and incubated for another 30 min. Then centrifugation at 10,000*g* for 10 min and the supernatants was saved as the mitochondria fraction. Protein concertation was detected by Bradford method [39].

Both fractions (40 μg/lane) were electrophoresed on 15% SDS polyacrylamide gels and transferred to nitrocellulose filter (Millipore, USA) on a Trans-Blot electrophoretic transfer tank (Bio-Rad, USA). Blots were blocked in TBS buffer (20 mM, pH 7.5 Tris-HCl buffer) containing 5% non-fat powdered milk and 0.05% Tween-20 overnight at 4 °C. Rabbit anti-cytochrome C polyclonal antibody (Boster, China) was diluted 300-fold in TBS containing 0.05% Tween-20 (TBST) for another night at 4 °C. Blots were washed with TBST three times. Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase was diluted 3000-fold in TBST, incubated for 2 h and then washed with TBST. The color was developed with 3′,3′-diaminobenzidine (DAB) coloration kit (CWBIO, China).

2.5. Detection of the mitochondrial membrane potential

1.5 mL cell suspension (about 1 × 10^6/mL) was added to 6-well plate, making the cell climbing slides [31]. Cells were pretreated with CsA or BCB (20 μM) for 3 h before CTD treatment. Processing intervals were 30, 60, 90 and 120 min, and Rhodamine 123 fluorescent probes (1 μM) (Sigma, USA) were added in darkness 10 min before the end of CTD processing. Cells were washed 3 to 5 times with phosphate buffer saline gently before observation. Fluorescence was measured by inverted fluorescence microscope with blue exciting light. Each group recorded at least 5 fields with fixed parameters (brightness 1.5, contrast 0.6 and gamma 0.7).

2.6. Detection of calcium concentration in mitochondria

Sf9 cell climbing slides were pre-treated with CsA or BCB for 3 h and then treated with CTD for 30, 60, 90 and 120 min. Calcein fluorescent probes (1 μM) (Molecular Probes, USA) were added to the culture plates 30 min before the termination, while $CoCl_2$ (10 mM) (Sigma, USA), a fluorescence quencher, 10 min before the ending. Following steps were same as above in Section 2.5.

2.7. Statistical analysis

According to Chen et al.'s [16] description of cell morphology after CTD treatment, normal cells showed clear edge and round shape and normal cell ratio (Normal cell ratio = normal cell number / total cell number × 100%) of each field was calculated. The activity of mitochondrial dehydrogenases (MDH) was reflected by the absorbance ($A = A_{test/control} - A_{blank}$). Relative fluorescent intensity (RFI) was calculated by Image-Pro Plus 6 (Media Cybernetics, USA). All experiments were performed at least three replicates and repeated at least two times. Representative experiments and mean values (±standard deviation, SD) were shown in figures. Statistical differences were determined by Least-Significant Difference with SPSS 19.0 (IBM, USA). Lowercase letters indicated significant difference between treatments ($P < 0.05$); Capital letters indicated a very significant difference ($P < 0.01$).

Please cite this article as: G. Cui, et al., Attribution of Bax and mitochondrial permeability transition pore on cantharidin-induced apoptosis of Sf9 cells, Pesticide Biochemistry and Physiology (2017), http://dx.doi.org/10.1016/j.pestbp.2017.01.010