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Staurosporine shows insecticidal activity against *Mythimna separata* Walker (Lepidoptera: Noctuidae) potentially via induction of apoptosis

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ABSTRACT

Staurosporine (STS), a wide-spectrum kinase inhibitor, is widely used in studies of apoptosis in mammalian cells. However, its physiological and mechanistic effects have never been clearly defined in insect cells, and other applications of STS have rarely been reported. The present study reveals the insecticidal activity of STS on larvae of *Mythimna separata* Walker, and the apoptotic mechanism induced by STS on lepidopteran Sf9 cell lines. We demonstrate that the viability of Sf9 cells is inhibited by STS in a time- and concentration-dependent manner. Intracellular biochemical assays show that STS-induced apoptosis of Sf9 cells coincides with a decrease in the mitochondrial membrane potential, the release of cytochrome c into the cytosol, a significant increase of the Bax/Bcl-2 ratio, and a marked activation of caspase-9 and caspase-3. These results indicate that a mitochondrial-dependent intrinsic pathway contributes to STS induced caspase-3 activation and apoptosis in Sf9 cells which is homologous to the mechanisms in mammalian cells. This study contributes to our understanding of the mechanism of insect cell apoptosis and suggests a possible new application of STS as a potential insecticide against Lepidopteran insect pests in agriculture.

1. Introduction

Mythimna separata Walker (*M. separata*, Lepidoptera: Noctuidae) is a major pest of cereal crops, particularly wheat, maize, and rice, throughout eastern China [1]. *M. separata* is widely used as a model insect organism in laboratory studies. In our previous work, we screened natural product compounds against *M. separata* for insecticidal activity. Among the compounds with identifiable insecticidal activity we found staurosporine (STS) to have high toxicity against the larvae of *M. separata*. STS, a potent protein kinase C (PKC) inhibitor, was first discovered in the extracts of *Streptomyces staurospores* [2]. However, despite initial hope for STS to be used as a therapeutic medicine with activity in killing tumorous cells, it was found to lack specificity. Subsequently, STS has been widely applied in mechanistic studies of apoptosis in mammalian cells including tumor cell lines [3], lymphocytes [4] and neuronal cells [5]. So far, only a very small number of reports have revealed insecticidal activity of STS and described the potential mechanisms of toxicity to insect cells.

As multicellular organisms, apoptosis is an essential biological process for insects. By targeting the mechanisms of apoptosis in insect cells it is possible to acquire new targets for insecticides. Apoptosis is a genetically programmed type of cell death mechanism [6]. It is a dynamic process that is essential to eliminate unwanted or abnormal cells and plays an important role in the stability of the internal environment and the balance of development in multicellular organisms [7,8]. Despite a wide range of inducing signals, some common pathways exist in apoptosis [9]. For instance, in the intrinsic pathway, mitochondria irreversibly commit cells to apoptosis by releasing death factors into cytosol [10]. Cytochrome c, a death factor, can form a complex with Apaf-1 in the presence of dATP in the cytosol. This is followed by activation of caspase-9 which results in the activation of a cascade of caspases [11]. Finally, these activated caspases degrade key structural and nuclear proteins and irreversibly commit the cells to death [12,13]. The distribution of cytochrome c between the cytosol and mitochondria changes after Bax translocates from the cytosol to the mitochondrial membrane [14].

STS-induced mammalian cell death has been shown to be associated with the mitochondrial apoptotic pathway [15,16], although the physiological and mechanistic significance of these changes have never been clearly defined in insect cells. Recently, some reports have provided contrasting evidence suggesting a possible role of mitochondrial factors during apoptosis in Drosophila [17,18]. Meanwhile, studies have also indicated that cytochrome c plays important roles in the apoptosis of lepidopteran cells such as *Spodoptera frugiperda* Sf9 cells, when induced by viral infections [19], ultraviolet radiation [20] and synthetic chemicals [21]. In our study, we have tried to work out if the mechanism of action of STS against Sf9 cells relies on the mitochondrial apoptotic pathway.

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This would not only supply evidence for identifying the apoptotic pathway used by Lepidoptera, but also make STS a new Lepidoptera-specific insecticide.

In this paper, we used different methods to verify the insecticidal activity of STS against Lepidoptera and show its ability to induce apoptosis in lepidopteran Sf9 cells. Our results indicate that STS can downregulate the mitochondrial membrane potential level, induce cytochrome c release, activate caspase-9 and caspase-3, and upregulate the expression level of Bax/Bcl-2. Taken together, this evidence shows that Sf9 cells were undergoing caspase-dependent mitochondrial apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Staurosporine (STS) was purchased from Abcam (Cambridge, MA, USA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), rhodamine123 (Rh-123), acridine orange (AO) and ethidium bromide (EB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Most antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) except for horseradish peroxidase (HRP)-conjugated anti-rabbit IgG which was from Sangon Biotech Co., Ltd. (Shanghai, China). The BCA Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). The caspase-3 Activity Assay Kit, caspase-9 Activity Assay Kit and the Mitochondria/Cytosol Fractionation Kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Other reagents and chemicals used were of analytical grade and purchased locally.

2.2. Cell culture and test insect

Spodoptera frugiperda (Sf9) cells obtained from American Type Culture Collection (ATCC) were maintained as monolayers in 25 cm^2 culture flasks at 27 °C in Serum-Free insect Cell Culture medium (Hyclone, Logan, Utah, USA) supplied with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Hyclone, Logan, Utah, USA).

Mythimna separata Walker (*M. separata*) was reared on maize leaves in glass containers (24 cm × 30 cm × 20 cm) at 25 ± 1 °C and 70% relative humidity (RH) under 12 h:12 h (Light: Dark). This strain has been laboratory-adapted for about 18 years, and never come into contact with insecticides. Third instar larvae were used for bioassays.

2.3. Test for insecticidal activity of staurosporine on M. separata Walker

The insecticidal activities of STS were measured against the newly molted third-instar larvae of *M. separata* according to a previously reported method [22]. Briefly, third-instar larvae of *M. separata* of the same size and level of health, and which had been starved for 6 h were fed with fresh chamber-grown corn leaves that had been dipped in 10, 20, 30, 40, 50 or 100 μg/mL STS solution (diluted with acetone) for 3 s and then taken out and dried at room temperature. The control group was fed with acetone-treated chamber-grown corn leaves. For each treatment, 30 larvae (10 larvae per group) were used, and the experiments were repeated 3 times. At 24 h and 72 h post treatment, the number of dead larvae in each group was recorded. The insecticidal activity of STS was estimated from the median dead concentration (LC_{50} value) of the test sample.

2.4. Cell viability assay

The effect of STS on the viability of Sf9 cells was determined by MTT assays [23]. In this assay, the yellow tetrazolium salt MTT was used as a substrate and reduced into purple formazan by mitochondrial succinate-dehydrogenase in viable cells only. Sf9 cell suspensions (2×10^5 cells/mL) were seeded onto 96-well plates (150 μL/well). The cells were grown for 24 h at 27 °C until they reached 2–4 × 10^6 cells/mL. Fifty microliters of STS was added to give final concentrations of 0.05, 0.1, 0.2, 0.5, 1 and 2 μM, and 0.1% DMSO was used as the control. After 12, 24 and 48 h of treatment, 20 μL of MTT (5 mg/mL) was added to each well. To dissolve the formazan crystals, the culture media was discarded and 150 μL of DMSO was added after 4 h incubation. Absorbance was measured at 492 and 630 nm by a Synergy H1 microplate reader (Bio-Teck, Winooski, VT, USA). Each experiment was repeated at least three times in quadruplicate. The inhibitory rates of the cells were calculated by the following formula: % inhibitory rate = [1 − (mean (OD_{492}-OD_{630}) in test wells) / (mean absorbency in control wells)] × 100%.

2.5. Apoptosis analysis by acridine orange (AO)/ethidium bromide (EB) assay

The dual staining of acridine orange (AO) and ethidium bromide (EB) was used to differentiate live cells from apoptotic and necrotic cells. All groups of cells were harvested and washed three times with PBS after being incubated with STS as described above, and then stained with AO and EB (to a final concentration 100 μg/mL for both) for 15 min in the dark. The morphology of the treated cells was examined by fluorescence microscopy (Lecois, DM3000), and 200 stained cells from each treatment group were counted.

2.6. Mitochondrial membrane potential ($\Delta\Psi m$) analysis

Mitochondrial membrane potential ($\Delta\Psi m$) is an important parameter of mitochondrial function. It is used as an early apoptotic marker in cells [24]. To determine whether mitochondrial damage occurs as an early event in STS-induced apoptosis of Sf9 cells, changes in $\Delta\Psi m$ were detected by fluorescence spectrophotometry in combination with the fluorescent marker Rhodamine123 (Rh-123). Rh-123 is a positively charged molecule which accumulates in the energized mitochondria. A decrease in the fluorescence intensity of Rh-123 indicates a decline in $\Delta\Psi m$. After being treated with STS at concentrations of 0.05, 0.1, 0.2, 0.5 and 1 μM for 5 h, Sf9 cells were harvested, washed twice with PBS (pH 7.4) and stained with Rh-123 for 20 min at 27 °C in the dark. Cells were collected by centrifugation after being washed three times with PBS to remove extracellular Rh-123. The fluorescence intensity was measured using flow cytometry (B.D. FACS Calibur) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data analysis was performed using the *Flowjo* software program.

2.7. Western blot analysis

Total protein from STS cells was extracted in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) with 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO, USA). Mitochondrial and cytosolic proteins were isolated using the Mitochondria/Cytosol Fractionation Kit according to the manufacturer's instructions, and quantified using the BCA method. Equal amounts of protein (20–40 μg) were separated by 8–15% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Atlanta, GA, USA). The blots were blocked in Tris-buffered saline-Tween (TBST; 10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Tween-20) with 5% non-fat dry milk at room temperature for 1 h. This was then processed by immunoblotting with primary antibodies for procaspase-3, cytochrome c, Bcl-2, Bax, and β-actin (diluted 1:1000; 1:200; 1:200; 1:200; 1:1000, respectively) with subsequent incubation with HRP conjugated secondary antibodies. Signals were visualized after treatment by enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA). All protein bands were scanned and integrated density values (IDVs) were quantified by *ImageJ* software and normalized to that of β-actin.