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Influence of cytochrome *c* on apoptosis induced by *Anagrapha* (*Syngrapha*) *falcifera* multiple nuclear polyhedrosis virus (AfMNPV) in insect *Spodoptera litura* cells

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Abstract

We investigated the influence of cytochrome *c* on apoptosis induced by *Anagrapha* (*Syngrapha*) *falcifera* multiple nuclear polyhedrosis virus (AfMNPV). Microscopic observation revealed that infection of SL-1 cells with AfMNPV resulted in apoptosis, displaying apoptotic bodies in fluorescent-stained nuclei of AfMNPV-infected SL-1cells. Western blot analysis demonstrated that AfMNPV-induced apoptosis in insect SL-1 cells was significantly inhibited by cyclosporin A which blocked a translocation of cytochrome *c* from the mitochondria to the cytosol. As determined by using AC-DEVD-AFC as substrate, the activity of caspase-3 in AfMNPV-induced cells was detected as early as 4 h post infection, gradually increased with time extension, and reached a highest level after 16 h of infection. However, activity of caspase-3 in apoptotic cells decreased in the presence of cyclosporin A (30 μM), indicating that activation of caspase-3 in SfaMNPV-induced cells was dependent on the release of cytochrome *c* from the mitochondria. In addition, cyclosporin A could markedly inhibit mitochondrial transmembrane potential ($\Delta\Psi m$) disruption in undergoing apoptotic cells. These data indicate that cytochrome *c* plays a key role in AfMNPV-induced apoptosis in *S. litura* cells and may be required for caspase activation during the induction of apoptosis.

Keywords: Cytochrome *c*; Caspase 3; Cyclosporin A; AfMNPV; *Spodoptera litura* cell line (SL-ZSU-1, SL-1)

1. Introduction

Apoptotic cell death is a fundamental feature of virtually all animal cells (Jacobson et al., 1997). It is an indispensable process during normal development, tissue homeostasis, development of the nervous system and the regulation of the immune system. Mitochondrion is an important organelle and its main function was thought to be limited to the production of energy in form of ATP. However, recent evidence is emerging that mitochondria participate in the central control or executioner phase of the cell death cascade (Susin et al., 1997; Kroemer et al., 1997; Desagher and Martinou, 2000; Gottlieb, 2000; Hengartner, 2000; Wang, 2001; Adrain and Martin, 2001). Mitochondria have been shown to play a critical role in the regulation of apoptosis process. Mitochondria contain key regulators of caspases, a family of proteases that are major factors in many apoptotic processes. The mitochondrial apoptosis pathway is mediated by cytochrome *c* release. Once released, cytochrome *c* together with deoxyadenosine triphosphate binds to apoptotic protease-activating factor-1 (Apaf-1), leading to an unmasking of its caspase recruitment domain and the subsequent binding and autoproteolytic activation of procaspase-9. The complex of procaspase-9, cytochrome *c*, and Apaf-1 are known as the apoptosome. Active caspase-9 then proteolytically activates downstream effector caspases, such as caspase-3, which degrades various cellular proteins propagating the apoptotic signal (Yang et al., 1997; Kluck et al.,

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1997; Zou et al., 1997; Nunez et al., 1998; Jiang and Wang, 2000; Joza et al., 2001).

Mitochondrial cytochrome *c*, which functions as an electron carrier in the respiratory chain, translocates to the cytosol in apoptotic cells, where it participates in the activation of specific caspases. Mitochondrial cytochrome *c* is a water-soluble protein, with a net positive charge, residing loosely attached in the mitochondrial intermembrane space and functions in the respiratory chain by interacting with redox partners of complex III and complex IV. Like most mitochondrial proteins, cytochrome *c* is encoded by a nuclear gene and synthesized as a cytoplasmic precursor molecule, apocytochrome *c*, which becomes selectively imported into the mitochondrial intermembrane space where a heme group is covalently attached via thioether linkages to cysteine residues, near the amino-terminus of apocytochrome *c* by the heme lyase (Dumont et al., 1987; Drygas et al., 1989; Stuart and Neupert, 1990). Many studies demonstrated that cytochrome *c* release from mitochondria is a key event and plays an important role in initiating apoptosis in the mammalian cells (Liu et al., 1996, 1997; Bossy-Wetzel et al., 1998; He et al., 2000; Arnoult et al., 2002). Sahdev et al. (2003) demonstrated the release of cytochrome *c* from mitochondria and consequently activation of caspase-3 during ultraviolet-inducing insect Sf9 cell apoptosis. The release of cytochrome *c* to cytosol might have the same pathway to activate caspase-3 both in insect and mammalian cells.

Based on the fact that AfMNPV infection has been previously shown to induce mitochondrial changes such as mitochondrial transmembrane potential ($\Delta\Psi m$) reduction, augmentation of cytochrome *c* in the cytosol and a corresponding decrease in the mitochondria in SL-1 cells (Xiu et al., 2005) and that cytochrome *c* release mediates mitochondrial apoptosis pathway via activation of caspase-3 (Sahdev et al., 2003), in the present study we attempted to investigate whether AfMNPV infection induces apoptosis in SL-1 cells. If it does, is AfMNPV-induced apoptosis related to cytochrome *c* pathway and does it involve activation of caspase 3? Our results indicated that the activity of caspase 3 was markedly inhibited by blocking the release of cytochrome *c* from mitochondria to cytosol and the morphological signs of apoptosis induced by AfMNPV was inhibited by cyclosporin A, suggesting that cytochrome *c* plays a key role in AfMNPV-induced apoptosis in *S. litura* cells and may be required for caspase activation during the induction of apoptosis.

2. Materials and methods

2.1. Cell, virus and chemicals

Spodoptera litura cell line (SL-1) was established by Xie et al. (1988) and was provided by Dr. Yang at the Institute of Entomology, Zhongshan University, P.R. China. *Anagrapha* (*Syngrapha*) *falcifera* multiple nuclear polyhedrosis virus (AfMNPV) was a gift of Dr. McIntoch of Department of Agriculture, USA. The cytochrome *c* monoclonal antibody anti-mouse (clone 7H8.2C12) was purchased from Becton Dickinson (BD). The polyclonal antibody anti-rabbit to caspase3 (H-277 sc-7148) was purchased from Santa Cruz Biotechnology, Inc.

2.2. Cell culture and virus infections

SL-1 cells were grown in Grace's medium (GIBCO) supplemented with 7% FBS, 0.3% yeast extract and 0.3% lactalbumin hydrolysate at 28 °C. For virus infection, 2×10^5 cells/ml were seeded into flasks or 6-well plates and incubated with AfMNPV for 1.5 h (MOI = 5). The virus inoculum was removed and cells washed twice with free-serum medium and then cultured in Grace's medium supplemented with 7% FBS. Cell numbers were determined using a hemacytometer and cell viability was determined using trypan blue exclusion.

2.3. Effect of cyclosporin A on apoptosis induced by AfMNPV in SL-1cells

To examine the potential role of cytochrome *c* on apoptosis in SL-1 cells infected by AfMNPV, 2×10^5 SL-1 cells were seeded into 6-wells plate, after cells attached the bottom of the plate, the medium was removed and cells were infected with AfMNPV at MOI of 5 and incubated for 1.5 h. Then virus inoculum was removed and medium with (30 μM) or without cyclosporin A (Calbiochem) was added to 6-wells respectively. After 16 h of infection, cells were fixed with 3.7% of formalin at 4 °C, dehydrated with ethanol and stained with DAPI for 30 min. The morphological changes of the nuclei of apoptotic cells were visualized by fluorescent microscopy.

2.4. Preparation of sub-fraction from SL-1 cells

SL-1 cells were harvested at 4 h intervals post infection by centrifugation at $800 \times g$ for 10 min and washed twice with ice-cold PBS. The cells were suspended in 200 μl modified lysis buffer (200 mM Hepes -KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA-Na_2, 1 mM DTT, 0.1 mM PMSF, 250 mM sucrose) and homogenized by a glass homogenizer. The homogenate was centrifuged at $750 \times g$ for 10 min at 4 °C to remove unbroken cells, large plasma membrane pieces and nuclei. The supernatant was further centrifuged at $10{,}000 \times g$ for 15 min. The supernatant and pellet were used to prepare a cytosolic fraction and the mitochondrial fraction respectively. The supernatant was centrifuged at $10{,}000 \times g$ for 1 h at 4 °C to remove the residue and saved as the cytosolic fraction. The pellet was resuspended in 100 μl lysis buffer, sonicated, then was subjected to centrifugation at $12{,}000 \times g$ at 4 °C for 1 h. The resulting supernatant was saved as the mitochondrial fraction. Both mitochondrial and cytosolic proteins were quantified and stored at −20 °C for western blotting.

2.5. DNA fragmentation assay

Genomic DNA was extracted from control and samples infected via AfMNPV for 16 h by using a standard phenol-chloroform method. The DNA samples were analyzed electrophoretically on 1.5% agarose gel and stained by ethidium bromide.

2.6. Western blot

Cytochrome *c* and caspase-3 changes were examined as described by Pervin et al. (2003). Briefly, both mitochondrial and cytosolic proteins were subjected to SDS-PAGE and subsequently transferred to nitrate cellulose membrane. The membranes were blocked with PBS containing 0.02% Tween-20 and 5% non-fat milk for 40 min. After washing three times with TBS, the membranes were incubated with respective primary antibodies (1:2000 dilution) in 1% BSA for 2–3 h. Following washing with TBS, the membranes were incubated with respective secondary antibody conjugated to horseradish peroxidase (1:200 dilution) for 1 h at room temperature. After washing three times, color development was carried out with 3,3′-diaminobenzidine as a substrate.