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Azadirachtin-induced apoptosis involves lysosomal membrane permeabilization and cathepsin L release in *Spodoptera frugiperda* Sf9 cells

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ABSTRACT

Azadirachtin as a kind of botanical insecticide has been widely used in pest control. We previously reported that azadirachtin could induce apoptosis of *Spodoptera litura* cultured cell line Sl-1, which involves in the up-regulation of P53 protein. However, the detailed mechanism of azadirachtin-induced apoptosis is not clearly understood in insect cultured cells. The aim of the present study was to address the involvement of lysosome and lysosomal protease in azadirachtin-induced apoptosis in Sf9 cells. The result confirmed that azadirachtin indeed inhibited proliferation and induced apoptosis. The lysosomes were divided into different types as time-dependent manner, which suggested that changes of lysosomes were necessarily physiological processes in azadirachtin-induced apoptosis in Sf9 cells. Interestingly, we noticed that azadirachtin could trigger lysosomal membrane permeabilization and cathepsin L releasing to cytosol. Z-FF-FMK (a cathepsin L inhibitor), but not CA-074me (a cathepsin B inhibitor), could effectively hinder the apoptosis induced by azadirachtin in Sf9 cells. Meanwhile, the activity of caspase-3 could also be inactivated by the inhibition of cathepsin L enzymatic activity induced by Z-FF-FMK. Taken together, our findings suggest that azadirachtin could induce apoptosis in Sf9 cells in a lysosomal pathway, and cathepsin L plays a pro-apoptosis role in this process through releasing to cytosol and activating caspase-3.

1. Introduction

Multiple regulatory factors associated with cell death were identified and proved to play important roles in apoptosis pathways. Recently, the lysosome has emerged as a key element in apoptosis pathways through selective lysosomal membrane permeabilization (LMP) and releasing proteases (Guicciardi et al., 2004; Kågedal et al., 2005). This apoptosis process can lead to several lysosomal changes, such as the increasing of lysosomal volume, secretion of proteases and total protease activity (Tardy et al., 2006), and changes in the subcellular localization of cathepsins B, D and L (Kirkegaard and Jäättelä, 2009). The cathepsin family of lysosomal proteases is an important hydrolase and is divided into cysteine, serine and aspartic cathepsins (Turk and Stoka, 2007; Turk et al., 2012). Especially, the cathepsins B, D and L are the most ubiquitous among the abundant lysosomal proteases (Rossi et al., 2004). In medical field, cathepsins are closely associated with many human diseases and their secretions could pose serious threats to human health. Many diseases, such as cancer, rheumatic arthritis, osteoarthritis, alzheimer, amyotrophy, multiple sclerosis and neuroblastoma, would occur with the secretion of cathepsins (Mort et al., 1984, 1998; Bever and Garver, 1995; Baici et al., 1995; Cataldo and Nixon, 1990; Sagulenko et al., 2008). In contrast to their tumor-promoting effects, there is also evidence to elucidate that they function as tumor suppressors (Berchem et al., 2002; Lopez-Otin and Matrisian, 2007; Hah et al., 2012; Hole et al., 2012; Marques et al., 2013). Regardless of the intensive studies done underlying the cathepsins, the role of cathepsins in cancer is still controversial and remained elusive.

In general, activation of the caspase cascade after LMP would activate the intrinsic pathway. Cathepsin B, the main lysosomal protease of the brain parenchyma, is shown increasing effect on

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caspase-11 and -1 activity, which plays important roles in brain ischemia by promoting both apoptotic and inflammatory processes (Foghsgaard et al., 2001). Furthermore, cathepsin B contributed to apoptosis via caspase activation in Dengue virus infection (Morchang et al., 2013). Many studies of cathepsin L on human diseases revealed the cysteine cathepsin L could also be associated with apoptosis process. Quantitative immunohistochemical analysis of human carotid atherosclerotic lesions suggested that the expression of cathepsin L in symptomatic patients was increased more than those who were asymptomatic patients (Li et al., 2009), though its detailed mechanism is poorly understood.

The fact that the cathepsins could be as an enhancer in apoptosis pathway triggered many attentions. Cathepsin D of the silkworm *Bombyx mori* (BmCatD) RNAi suggested that BmCatD is critically involved in the programmed cell death of the larval fat body and gut in silkworm metamorphosis (Gui et al., 2006). Moreover, many investigations showed that cathepsins, especially cathepsin B and L, were activated in the process of ovigenesis and caused the degradation of ootid (Matsumoto et al., 1997; Uchida et al., 2001, 2004). As such strategy underlying cathepsins-induced cell death could bring novel insight into control some important pests, such as *Rhodnius prolixus* and *Ceratitis capitata* (Ferreira-DaSilva et al., 2000; Rabossi et al., 2004). Thus, understanding the roles of cathepsins in apoptosis could provide a new prevention strategy for pest management.

As a kind of botanical insecticide mainly extracted from the neem tree *Azadirachta indica* (A.Juss), azadirachtin (AZA) has attracted widely attention for pest control in the last few years (Isman et al., 1990; Schmutterer, 1990; Linton et al., 1997). Such semiochemical properties equipped strong antifeedant activities against many insect species, which is also companied by remarkable insect growth regulatory (IGR) activities and sterility effects (Mordue and Blackwell, 1993; Huang et al., 2004; Tan and Luo, 2011). Apoptosis can be induced by particular endogenous and exogenous factors (Arena et al., 1992). A great deal of research at the cellular level revealed that azadirachtin could affect cell proliferation and induce apoptosis (Salehzadeh et al., 2003; Anuradha et al., 2007; Kumar et al., 2007). Our previous studies showed that P53 protein was involved in cell cycle arrest, apoptosis induction and cell proliferation inhibition when the *Spodoptera litura* Sl-1 cells were treated with azadirachtin (Huang et al., 2011). Apart for that, the comprehensive understanding still requires more studies underlying other possible signal factors participating in AZA-induced apoptosis in insect cultured cells. Here, we analyzed the cell proliferation inhibition and morphological changes of lysosomes by using fluorescence microscope and flow cytometry methods during AZA-induced apoptosis. More importantly, we further proved the effects of cathepsin B (SCB) and cathepsin L (SCL) on Sf9 cells apoptosis exerted through the interferencing of activity inhibitors CA-074me (CA) and Z-FF-FMK (FF). Our data showed that lysosomal changes occurred in AZA-induced apoptosis and SCL activity had important repercussions in the sensitivity of Sf9 cells to AZA, which could have promotion implications.

2. Materials and methods

2.1. Cell line and culture conditions

Spodoptera frugiperda Sf9 cells were cultivated at 27 °C in 25 cm^2 flasks in 3 mL Grace's insect cell culture medium (Gibco, USA) containing 10% fetal bovine serum (FBS), 0.3% yeast extract, 0.3% lactalbumin hydrolysate and 0.3% peptone. Cells were seeded and planked with 0.5×10^6–1×10^6 cells/mL density in 35 mm cultural plates for 24 h when the cells were at the optimum conditions for the following treatment.

2.2. Evaluation of cells viability by 3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl (MTT) assays

Cells in good condition were selected and incubated for 24 h at 27 °C in the 96-well plates with 100 μL cell suspensions in each hole. Moderate quantity of AZA (the final concentration of 0.75 μg/mL dissolved in 0.1% dimethylsulforxide (DMSO)) was added into the cell suspensions. 0.1% DMSO was used as the control. After different treatment times (0, 12, 24, 36, 48 and 60 h, respectively), 10 μL freshly prepared MTT was added and the plates were incubated in the darkness for 4 h at 27 °C. Then discarded the medium, and added 150 μL fresh DMSO to each well to dissolve the formazan crystal by orbital shaking in the darkness for 15 min. The absorbance was measured at 570 nm by a microplate reader (BioTek, USA). Cell viability was calculated by the equation as: Cell viability (%) = $(OD_{treatment}/OD_{control}) \times 100\%$.

2.3. Morphological observation

2.3.1. Cell morphological observation

Cells with the density of 0.5×10^6–1×10^6 cells/mL were collected and incubated for 24 h in 12-well plates. Then cells at proliferation phase were treated with AZA for 0, 12, 24, 36, 48 and 60 h, respectively. Morphological characteristics of Sf9 cells were recorded with inverted phase contrast microscope (IPCM) (Olympus, Japan). Ultrastructure of cells were obtained by transmission electron microscope (TEM), the treated cells and control group were washed with 0.1 M PBS (phosphate buffer saline) three times before being fixed in 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M PBS buffer pH 7.2 for 2 h. After incubation time, they were rinsed with 0.1 M PBS and post-fixed in 1% osmium tetraoxide at room temperature for 1 h. All samples were washed three times with 0.1 M PBS following dehydration through an alcohol series and embedded in spur resin. The specimens were ultra sectioned to 60 nm thickness, stained with 0.5% uranyl acetate and lead citrate, and finally examined under TEM (TECNAIG^{2}12).

2.3.2. 4′,6-Diamidino-2-phenylindole (DAPI) staining analysis

Cells were seeded in 12-well plates, and exposed to 0.75 μg/mL AZA for 0, 12, 24, 36, 48 and 60 h, respectively, and then the nutrient supernatant was discarded. The uncovered cells were fixed in 4% paraformaldehyde for 15 min and washed with PBS twice. Finally, cells were stained in DAPI dye liquor (Southern Biotech Company, USA) with the final concentration of 1 mg/L for 15 min and washed in PBS once again. The samples were observed and photographed by fluorescence microscope (FM) (Olympus BX51, Olympus, Japan).

2.3.3. Lyso-Tracker Red probe staining analysis

Cells were treated with AZA for different times (0, 12, 24, 36, 48 and 60 h, respectively), and incubated with 500 μL pre-incubated Lyso-Tracker Red fluorescent probe (diluted 1:15,000) at 27 °C for 2 h, then removed the nutrient supernatant with fluorescent probe. The fresh nutrition solution was added and the lysosomes morphology changes were showed by FM (Olympus BX51, Olympus, Japan). For quantify the fluorescent value, the sample was mixed by successive aspiration with pipette for several times, then immediately transferred into black polystyrene 96-well plates. The relative fluorescence units of probe were read by fluorescence microplate reader (FMR) with excitation/emission wavelengths at 577 nm/590 nm (BioTek, USA).

2.3.4. Acridine orange (AO) staining analysis

Cells were seeded in 6-well plates, and exposed to AZA for different times. Cells were then collected, and resuspended by PBS. Cell suspension were dyed by AO staining with the concentration of 1 μM and incubated for 15 min in the darkness. Cover slips were