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Apoptosis-suppressing and autophagy-promoting effects of calpain on oridonin-induced L929 cell death

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ABSTRACT

Calpain, calcium-dependent cysteine protease, is reported here to impose the crucial influence on oridonin-induced L929 cell apoptosis and autophagy. We found that inhibition of calpain increased oridonin-induced Bax activation, cytochrome *c* release and PARP cleavage, indicating that calpain plays an anti-apoptotic role in oridonin-induced L929 cell apoptosis. To explore this potential anti-apoptotic mechanism, we inhibited calpain and proteasome activity in oridonin-induced L929 cell apoptosis, and discovered that the inducible IκBα proteolysis was partially blocked by the inhibition of either calpain or proteasome, but completely blocked by the inhibition of both. It demonstrated that calpain and proteasome were two distinct pathways participating in IκBα degradation. To further study the role of calpain in oridonin-induced L929 cell autophagy, we discovered that calpain inhibitor decreased oridonin-induced autophagy, as well as Beclin 1 activation and the conversion from LC3-I to LC3-II. Moreover, Inhibition of autophagy by 3-MA increased oridonin-induced apoptosis. In conclusion, besides suppressing apoptosis, calpain promotes autophagy in oridonin-induced L929 cell death, and inhibition of autophagy might contribute to up-regulation of apoptosis.

Programmed cell death (PCD), a critical mechanism for development and homeostasis of multicellular organisms, consists of two main forms: apoptosis and autophagy [1]. Apoptosis (type I PCD) is a cell-intrinsic suicide mechanism regulated by variously cellular signaling pathways [2]. Defective apoptotic cell death can result in autoimmune diseases and tumorigenesis, whereas superabundant apoptosis is often associated with neurodegenerative diseases [3]. Autophagy (type II PCD) has multiple physiological functions in multicellular organisms, including lysosome-dependent protein degradation and organelle turnover [4]. It is not only a survival response to either growth factor or nutrient deprivation but a mechanism for tumor cell suicide induced by chemotherapy or radiation [5,6]. Recent investigations have demonstrated that the co-regulation of both apoptosis and autophagy may participate in mammalian cell death [7]. Meanwhile, other studies have further pointed out that apoptosis and autophagy may be interconnected and even simultaneously regulated by the same trigger [8]. Due to the cellular context and stimulus, the execution of apoptosis is preceded by and even depends on the autophagic occurrence [9,10]. Moreover, some studies have reported that autophagy can resist or suspend apoptosis [11]. Accordingly, under some situations, there are multiple connections between apoptotic and autophagic processes that can together seal the fate of cells [12].

Calpain is calcium-dependent intracellular cysteine protease that plays a crucial role in the regulation of cell spreading, cell migration, programmed cell death and cell cycle progression [13,14]. Calpain mediated cleavage can regulate the activity of diverse substrates, such as transcription factors, cytoskeletal proteins, kinases and apoptotic proteins [15–17]. Moreover, calpain is correlated with the endoplasmic reticulum and Golgi that are likely reservoirs for autophagosome membranes [18]. It can also be activated by several stimuli that trigger autophagy. Nevertheless, many of recent reports have demonstrated that calpain plays a pivotal pro- or anti-apoptotic role in cell death signaling pathways [19–22]. However, their participating mechanisms still remain ambiguous. Therefore, it seems to be important to elucidate what role calpain can play in such pathways.

Oridonin (shown in Fig. 1), an active diterpenoid isolated from *Rabdosia Rubescens*, has been traditionally and widely used for treatment of various human diseases due to its uniquely biological, pharmacological and physiological functions (e.g., anti-tumor, anti-inflammation and anti-bacterial) [23,24]. Therefore, oridonin would be used to explore more significant molecular mechanisms of programmed cell death as an important study model.

In our previous studies, oridonin was found to induce murine fibrosarcoma cell (L929 cell) apoptosis through mitochondrial

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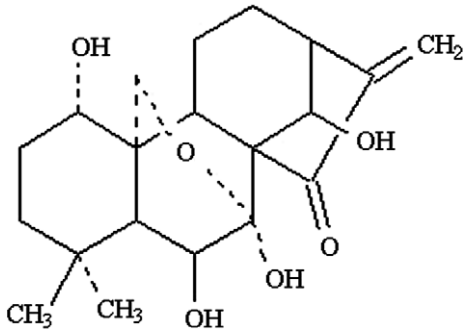

Fig. 1. Chemical Structure of oridonin.

and ERK signal pathways [25]. Interestingly, we also found that caspase, a mediator of apoptosis triggered by extracellular stimuli, did not mediate apoptosis, but protected L929 cells from oridonin-induced cell death [26]. Moreover, for many researchers, another intriguing aspect of calpain is to further investigate its potent biological implications in autophagic pathways [27]. Driven by the above-mentioned interesting phenomena, we further investigated the effects of calpain in oridonin-induced L929 cell apoptosis and autophagy for further understanding of calpain's role in cell death pathways.

Here, we initially found that calpain played an anti-apoptotic role in the oridonin-induced L929 cell apoptosis. According to the further study of calpain in oridonin-induced autophagy, we discovered that calpain promoted autophagy. Moreover, in the study of the connection between apoptosis and autophagy, we concluded that inhibition of autophagy might lead to up-regulation of apoptosis.

Materials and methods

Reagents

Oridonin was obtained from the Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China). The purity of oridonin was measured by HPLC and determined to be 99.4%. Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.1% in cell culture and did not exert any detectable effect on cell growth or cell death. Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); monodansylcadaverine (MDC), autophagy inhibitor 3-methyladenine (3-MA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PI3K family inhibitor wortmannin, calpain inhibitor ALLM (*N*-Acetyl-L-leucyl-L-leucyl-L-methioninal), NF-κB inhibitor PDTC, proteasome inhibitor MG132 and acridine orange (AO) were purchased from Sigma Chemical (St. Louis, MO, USA). Pan-caspase inhibitor (z-VAD-fmk) was purchased from Enzyme Systems (Livermore, CA, USA). Polyclonal antibodies against Bax, Bcl-2, Bcl-X_L, cytochrome *c*, poly-(ADP-ribose) polymerase (PARP), IκB, phosphorylated IκB, Akt, phosphorylated Akt, LC3, Beclin, β-actin and horseradish-peroxidase-conjugated secondary antibodies (goat-anti-rabbit or goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The murine fibrosarcoma L929 (#CRL-2148) cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Shengma Yuanheng, Beijing, China), 10 μg/ml streptomycin, 100 U/ml penicillin and 0.03% L-glutamine and maintained at 37 °C with 5% CO_2 at a humidified atmosphere.

Growth inhibition assay

The cytotoxic effect of oridonin in L929 cells was measured by MTT assay as described elsewhere [28]. The L929 cells were incubated in 96-well tissue culture plates (NUNU, Roskilde, Denmark) at a density of 5×10^3 cells/well. After 24 h incubation, the cells were treated with or without z-VAD-fmk, ALLM, PDTC, MG132 or wortmannin at the given concentrations for 1 h and subsequently treated with oridonin for different time periods. The cytotoxic effect was measured with a plate reader by MTT assay. The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell growth inhibition } (\%) = [A_{492}(\text{control}) - A_{492}(\text{oridonin})]/[A_{492}(\text{control}) - A_{492}(\text{blank})] \times 100$$

Observation of morphological changes

L929 cells were seeded into 6-well culture plates with or without oridonin and cultured for 24 h. The cellular morphology was observed by using a phase contrast microscopy (Leica, Nussloch, Germany).

Fluorescence morphological examination

Apoptotic nuclear morphology was assessed by staining the cells with the fluorescent DNA-binding dye AO. The cells were harvested and incubated with 50 μmol/L oridonin, washed with PBS for three times and then stained with 20 μg/ml AO for 15 min. After staining, the color and structure of the different cell types were observed under a fluorescence microscope (Olympus, Tokyo).

Flow cytometric analysis of autophagy

L929 cells were pretreated with 3-MA or ALLM for 1 h before the addition of oridonin. After 24 h, the cells were harvested and rinsed with PBS two times by centrifugation at 1000*g*. For measuring autophagy, the cell pellet was suspended with 0.05 mmol/L MDC at 37 °C for 1 h as described previously [29], and then the samples were analyzed by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA, USA) to determine the percentage of cells undergoing autophagy.

LDH activity-based cytotoxicity assays

The LDH (lactate dehydrogenase) activity was assessed using a standardized kinetic determination (Zhongsheng LDH kit, Beijing, China). LDH activity was measured in both floating dead cells and viable adherent cells. The floating cells were collected from the culture medium by centrifugation (240*g*) at 4 °C for 5 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp) [30]. The LDH released in the culture medium (LDHe) was used as an index of necrotic death, and the LDH present in the adherent viable cells was designated as intracellular LDH (LDHi). The percentage of apoptotic cell deaths was calculated as follows:

$$\text{Apoptosis } \% = \text{LDHp}/(\text{LDHp} + \text{LDHi}) \times 100$$

Western blot analysis

L929 cells were treated with 50 μmol/L oridonin for 6, 12, 24 and 36 h, respectively. Both adherent and floating cells were collected, and then Western blot analysis was carried out as previously described [31]. Briefly, the cell pellets were resuspended with lysis buffer consisting of Hepes 50 mmol/L PH 7.4, Triton X-100 1%, sodium orthovanada 2 mmol/L, sodium fluoride 100 mmol/L, edetic acid 1 mmol/L, PMSF 1 mmol/L, aprotinin (Sigma, MO, USA) 10 mg/L and leupeptin (Sigma) 10 mg/L and lysed at 4 °C for 1 h. After 12,000g centrifugation for 15 min, the protein content of supernatant was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of the total protein were separated by 12% SDS–PAGE and transferred to nitrocellulose membranes, the membranes were soaked in blocking buffer (5% skimmed milk). Proteins were detected using polyclonal antibodies and visualized using anti-rabbit or anti-mouse IgG conjugated with peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

Statistical analysis

All the presented data and results were confirmed in at least three independent experiments. The data are expressed as means ± SD. Statistical comparisons were made by Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Oridonin-induced apoptotic cell death in L929 cells

Oridonin inhibited L929 cell growth in a time- and dose-dependent manner. The IC_{50} for 24 h oridonin treatment was 54.3 μmol/L (Fig. 2A). To determine the features of oridonin-induced L929 cell growth inhibition, the morphologic changes of cell nuclei was examined. Compared with the control group, remarkable morpho-