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Autophagy inhibition enhances silibinin-induced apoptosis by regulating reactive oxygen species production in human prostate cancer PC-3 cells

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ABSTRACT

Silibinin is a major bioactive component of silymarin and has anticancer effects on cancer cell line and has been used as a supportive therapy for chronic inflammatory liver condition. These anticancer effects of silibinin have been demonstrated both *in vitro* and *in vivo* cancer models. Although various evidences showed apoptosis signaling pathways by silibinin, there is no report to address the clearly mechanism of silibinin-induced autophagy in prostate cancer PC-3 cells. Our study showed that silibinin triggered autophagy through up-regulation of microtubule-associated protein 1 light chain 3 (LC3)-II, formation of acidic vesicular organelles (AVO) and punctuate of GFP-LC3, which was inhibited by 3-methyladenine (3-MA), an inhibitor of specific autophagy. In addition, silibinin induced autophagy through production of reactive oxygen species (ROS). Inhibition of ROS with diphenyleneiodonium (DPI), a ROS inhibitor, attenuated silibinin-triggered autophagy. Inhibition of autophagy with 3-MA enhanced the silibinin-induced apoptosis through the regulation of caspase-3 and PARP. These results suggested that silibinin induced autophagy by regulating ROS and its mechanism played a protective role against apoptosis in PC-3 cells.

1. Introduction

Prostate cancer is the second leading cause of cancer-related deaths among men in the United States, and the increase of incidence and mortality for prostate cancer has been estimated in the worldwide [1,2]. Unfortunately, molecular mechanism underlying progression of prostate cancer is not exactly being revealed, but aging, genetics and westernized diet are the important risk factors. At initial stages, surgical prostatectomy, radiotherapy and hormonal therapy are most effective methods [3,4]. However, an androgen-independent stage of malignancy on aggressive and spreading is difficult to treatment [5,6]. Therefore, more effective strategies must be necessary to development for novel therapeutic targets and molecular regulatory agents.

Silibinin is a major bioactive component of silymarin extracted from milk thistle and has been used as a supportive therapy for chronic inflammatory liver condition. Recently, anticancer effects of silibinin have been demonstrated in various human cancers such as prostate, breast, skin, colon, ovarian and lung cancer in both *in vitro* and *in vivo* models [7–9]. On the other hand, it has been reported that silibinin is used as a potential drug for treatment of Alzheimer's disease (AD) through amelioration of oxidative stress and inflammatory response [10]. In addition, many studies of silibinin have focused on relationship between apoptosis and autophagy in some cancer cells. However, its mechanism on the autophagy induced by silibinin remains unclear.

Autophagy is an intracellular catabolic process that plays a critical role in degradation of cytoplasmic proteins [11,12]. When autophagy is induces, the formation of autophagosomes is initiated by induction of autophagy genes including microtubule-associated protein 1 light chain 3 (LC3), phosphatidylinositide 3 kinase (PI3K), Beclin-1 and autophagy genes (ATGs) [13]. Induction of autophagy is involved with various factors and signaling pathway in different cellular environment. Among them, reactive oxygen species (ROS)

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are presented to be important signals to activate autophagy as well as apoptosis by a variety of stimulating conditions, suggesting that it may have synergetic effects in autophagy and apoptosis [14]. Generation of ROS including superoxide anion, hydroxyl radical and hydrogen peroxide is associated with cellular metabolism [15]. These ROS can be stimulated autophagy through the regulation of LC3, Beclin-1 and ATGs in cancer cells. In some studies, during amino acid starvation, ROS generated from mitochondria can directly modulate the autophagy.

In this study, we investigated that silibinin induced autophagy in PC-3 cells. In addition, ROS generation by silibinin regulates autophagic pathway, which serves as a protect role against silibinin-induced apoptosis.

2. Materials and methods

2.1. Reagents and antibodies

Propidium iodide (PI), 3-methyadenine (3-MA), acridine orange (AO) and 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diphenyleneiodonium (DPI) was purchased from Calbiochem (Merck, Darmstadt, Germany). FITC Annexin-V apoptosis Detection kit was purchased from BD Bioscience (San Jose, CA, USA). The ECL Western Kit was purchased from iNtRON Biotechnology (Seongnam, Korea). Antibodies for Caspase-3 and β-Actin were purchased from Santa Cruz Biotechnology. Antibodies for poly (ADP-ribose) polymerase-1 (PARP-1) and LC3 were purchased from Cell Signaling.

2.2. Cell lines and cell culture

Human prostate cancer PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). PC-3 cells were cultured in Dulbecco's modified Eagle's minimal medium (DMEM, WelGENE Inc., Korea) supplemented with 10% FBS and 1% penicillin-streptomycin solution with 5% CO_2 at 37 °C.

2.3. Western blotting

Cell extracts were prepared by incubating the cells in lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 20 mg/ml aprotinin, 50 μg/ml leupetin, 1 mM benzamdine, 1 mg/ml pepstatin]. Forty micrograms of proteins determined by the BSA method were electrophoretically separated using 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in TBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] at room temperature for 1 h. The membranes were incubated with primary and secondary antibodies. The membranes were then washed 3 times with TBS-T buffer for 10 min. Finally, the membranes were detected with an ECL western blotting detection reagent (iNtRON Biotechnology, Seongnam, Korea). The densities of each band were determined with a fluorescence scanner (LAS 3000, Fuji Film, Tokyo, Japan) and analyzed with Multi Gauge V3.0 software.

2.4. Detection of autophagic vacuoles by acridine orange (AO)

PC-3 cells were seeded at a density of 1×10^5/ml in a 6-well culture dish and incubated for 24 h. After treatment with 150 μM of silibinin for 48 h in the presence or absence of 3-MA, cells were stained with 1 μg/ml AO in serum-free medium for 15 min, then washed twice with PBS. Images of AO staining were visualized immediately using confocal microscope (Olympus, Tokyo, Japan). The cytoplasm and nucleus of cells fluoresced bright green,

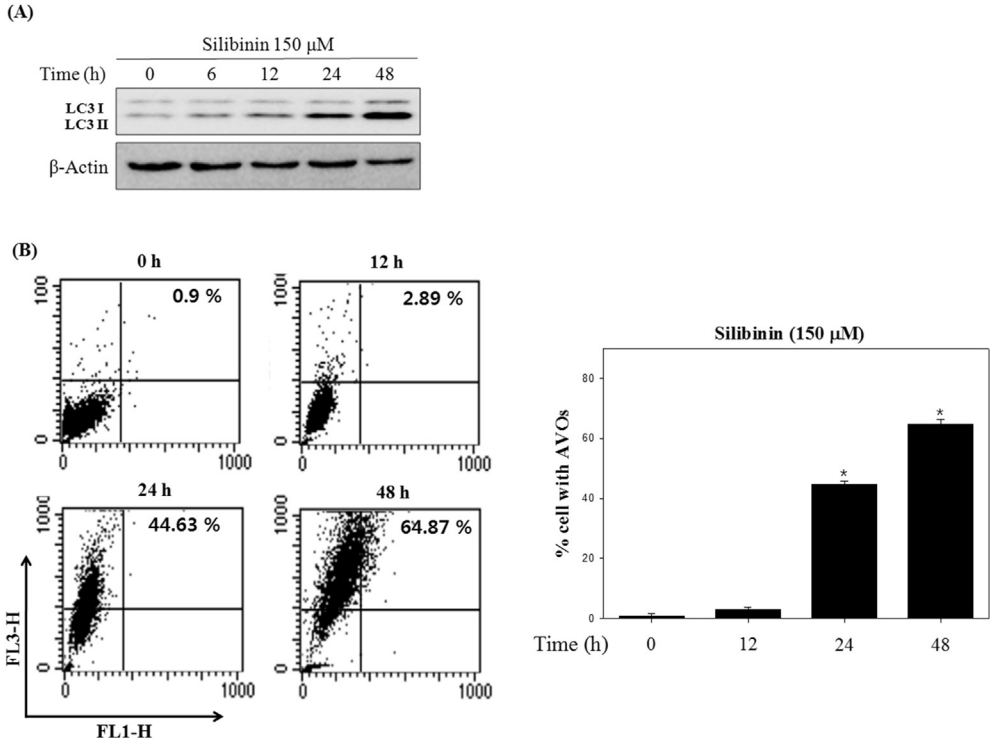

Fig. 1. Silibinin induced autophagy in PC-3 cells (A) LC3-II protein, a known autophagy marker, was detected by western blotting. PC-3 cells were treated with 150 μM silibinin for indicated times. β-Actin was used as a loading control. (B) Quantification of acidic vesicular organelles (AVO) accumulation was calculated by FL-3 channel of flow cytometry. PC-3 cells were treated with 150 μM silibinin for indicated times. Then cells were incubated with 1 μg/ml acridine orange (AO) in serum-free medium for 15 min. Data are presented as mean ± SD (n = 3 in each group). *$p < 0.001$ vs. the control group.