Silibinin Induces Apoptotic Cell Death Via ROS-dependent Mitochondrial Pathway in Human Glioma Cells

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It has been reported that silibinin, a natural polyphenolic flavonoid, induces cell death in various cancer cell types. However, the underlying mechanisms by which silibinin induces apoptosis in human glioma cells are poorly understood. The present study was therefore undertaken to examine the effect of silibinin on glioma cell apoptosis and to determine its underlying mechanism in human glioma cells. Apoptosis was estimated by FACS analysis. Reactive oxygen species (ROS) generation and mitochondrial membrane potential (Ψm) were measured using fluorescence dyes DCFH-DA and $DiOC_6(3)$, respectively. Cytochrome c release from mitochondria and caspase-3 activation were estimated by Western blot analysis using specific antibodies. Exposure of cells to 30 mM silibinin induced apoptosis starting at 6 h, with increasing effects after 12-48h in a time-dependent manner. Silibinin caused ROS generation and disruption of ym, which were associated with the silibinin-induced apoptosis. The silibinin-induced ROS generation and disruption in ym were prevented by inhibitors of mitochondrial electron transport chain. The hydrogen peroxide scavenger catalase blocked ROS generation and apoptosis induced by silibinin. Silibinin induced cytochrome c release into cytosolic fraction and its effect was prevented by catalase and cyclosporine A. Silibinin treatment caused caspase-3 activation, which was inhibited by DVED-CHO and cyclosporine A. Pretreatment of caspase inhibitors also protected against the silibinin-induced apoptosis. These findings indicate that ROS generation plays a critical role in the initiation of the silibinin-induced apoptotic cascade by mediation of the mitochondrial apoptotic pathway including the disruption of Ψm, cytochrome c release, and caspase-3 activation.

Key words : silibinin, apoptosis, mitochondrial membrane potential, caspase, cytochrome c, human glioma cells

Introduction

Glioblastoma is the most lethal and frequent primary tumors that arise in the brain, with a median survival of less than 1 year[1]. It is comprised of poorly differentiated heterogeneous neoplastic astrocytes with aggressive proliferation and highly invasive properties. After diagnosis of glioblastioma, the median survival time of 9-12 months has remained unchanged despite aggressive treatment including surgery, radiation, and chemotherapy[2,3]. Thus, need to develop new effective strategies for controlling glioblastoma is particularly high. Because glioblastoma cells avoid differentiation and apoptosis, the induction of differentiation and apoptosis in glioblastoma cells may be considered as a potential treatment strategy.

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Flavonoids and other polyphenolic compounds constitute one of the most numerous groups in the plant kingdom and have recently received much attention as potential chemopreventive and chemotherapeutic agents[7-9]. Considering that many chemotherapeutic agents have serious side effects and development of multidrug resistance further limits chemotherapy in cancer, flavonoids may be a very promising group of compounds exerting the chemopreventive and chemotherapeutic effects. Previous studies have shown that flavonoids induces growth inhibition and cell death in human glioma cells[7-9].

Silibinin, a natural polyphenolic flavonoid, is a major bioactive component of silymarin which is isolated from the plant milk thistle (Silybummarianum), and has been extensively used for its hepatoprotective effects in Asia and Europe. Recently, anticancer activities of silibinin have been demonstrated in various cancers including prostate cancer in both in vitro and in vivo models[10-13]. Silibinin has also been reported to sensitize human glioma cells to TRAIL-mediated apoptosis[14]. However, the effect of silibinin on glioma cell

death and the underlying mechanisms of action are poorly understood.

The present study was undertaken to determine the molecular mechanisms by which sililbinin induces apoptosis in human glioma cells. Our data demonstrated that silibinin induces human glioma cell death via a caspase-dependent mitochondrial pathway. These results suggest that silibinin may be an effective agent for both prevention and intervention of human glioblastomas.

Materials and Methods

1. Reagents

Trolox, catalase, cyclosporine A, antimycin A, rotenone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, and propidium iodide were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Tween 20, z-VAD-FMK, and DEVD-CHO were purchased from Calbiochem (San Diego, CA, USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and $DiOC_6(3)$ were obtained from Molecular Probes (Eugene, OR, USA). Antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals were of the highest commercial grade available.

2. Cell culture

U87MG cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Invitrogen, Carsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA) at 37℃ in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsinsolution. The cells were grown on well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved. Unless otherwise stated, cells were treated with silibinin in serum-free medium. Test reagents were added to the medium 30 min before silibinin exposure.

3. Measurement of apoptosis

Apoptosis was estimated by cell cycle analysis. Cells were grown in 6-well plates and were treated as indicated. Then, attached and floating cells were pooled, pelleted by centrifugation, washed in PBS, and fixed with cold 70% ethanol containing 0.5% Tween 20 at 4℃ overnight. Cells were washed and resuspended in 1.0 ml of propidium iodide solution containing 100 mg of RNase A/ml and 50 mg propidium iodide/ml and incubated for 30 min at 37℃. Apoptotic cells were assayed using FACSort Becton Dickinson Flow Cytometer at 488 nm and data were analyzed with CELLQuest Software. Cells with sub-G1 propidium iodide incorporation were considered as apoptotic. The percentage of apoptotic cells was calculated as the ratio of events on sub-G1 to events from the whole population.

4. Measurement of reactive oxygen species (ROS)

The intracellular generation of ROS was measured using DCFH-DA. The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. The probe (DCFH) is rapidly oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells cultured in 24-well plate were preincubated in the culture medium with 30 mM DCFH-DA for 1 h at 37℃. After the preincubation, the cells were exposed to 30 μM silibinin for various times. Changes in DCF fluorescence was assayed using FACSort Becton Dickinson Flow Cytometer (Becton-Dickinson Bioscience, San Jose, CA, USA) and data were analyzed with CELLQuest Software.

5. Measurement of mitochondrial membrane potential (Ψm)

The mitochondrial transmembrane potential was measured with $DiOC_6(3)$, a fluorochrome that is incorporated into cells depending upon the Ψm15. Loss in $DiOC_6(3)$ staining indicates disruption of the mitochondrial inner transmembrane potential. Cells were stained with $DiOC_6(3)$ at a final concentration of 50 nM for 20 min at 37℃ in the dark. Cells were washed and resuspended in Hank's balanced salts solution containing Ca^{2+} and Mg^{2+}. Thef luorescence intensity was analyzed with a FACScan flow cytometer using the fluorescence signal 1 channel.

6. Western blot analysis

Cells were harvest at various times after silibinin treatment and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). Cell debris was removed by centrifugation at 10,000 g for 10 min at 4℃. The resulting supernatants were resolved on a 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with different primary antibodies. The membranes were washed and incubated with horseradish

peroxidase-conjugated secondary antibodies. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

7. Measurement of caspase-3 activity

Caspase-3 activation was estimated by measuring expression levels of procaspase-3. Cells were exposed to silibinin for various times and expression of porcaspase-3 was evaluated by Western blot analysis using specific procaspase-3 antibody as described above.

8. Measurement of cytochrome c release

Cells were harvested and washed twice with PBS. The Cells were incubated with extraction buffer (10 mM Hepes, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.05% digitonin, and 1 mM phenylmethylsulfonyl fluoride) at 4℃ for 10 min, then centrifuged at 100000 g for 10 min at 4℃. The supernatant represented the cytosolic protein. The fraction was loaded onto a 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking in 5% non-fat dried milk at room temperature for 30 min, membranes were probed with rabbit polyclonal anti-cytochrome, followed by horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using the ECL detection system (Amersham, Buckinghamshire, UK).

9. Statistical analysis

The data are expressed as means±SEM and the difference between two groups was evaluated using Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. A probability level of 0.05 was used to establish significance.

Results

1. Effect of silibinin on induction of apoptosis

To ascertain whether silibinin induces apoptosis, cells were exposed to 30 mM silibinin for 24 hr and apoptosis was estimated by flow cytometric analysis. The DNA content analysis by flow cytometry showed that the proportion of the cells in the sub-G1 phase (apoptotic cells) was increased 3.90% of control to 32.95% after silibinin treatment (Fig. 1A). Silibinin caused apoptosis in a time-dependent manner (Fig. 1B).

2. Role of ROS generation in silibinin-induced cell death

To determine whether silibinin induces ROS generation in human glioma cells, the cells were exposed to silibinin and changes in DCF fluorescence were measured using flow cytometry. Silibinin caused an increase in ROS generation after 6 h of silibinin treatment as evidenced by the increase in M1 region (Fig. 2A). Silibinin caused ROS generation as early as 1 h after treatment and increased in a time-dependent fashion up to 6 h and remained unchanged to 24 h (Fig. 2B). It has been suggested that the ROS generation is regulated by a mitochondrial electron transport chain16. To examine the role of the mitochondrial transport chain system in the silibinin-induced ROS generation, the effects of cyclosporine A, an inhibitor of mitochondrial permeability transition pore, and antimycin A and rotenone, inhibitors of mitochondrial electron transport chain complexes I and III, on the ROS generation. Silibinin did not cause ROS generation in the presence of these inhibitors (Fig. 2C). The treatment with these inhibitors alone did not significantly affect the levels of ROS as compared with that in untreated control cells. We then evaluated the effects of catalase and cyclosporine A on the silibinin-induced apoptosis. The results of Fig. 2D show that the silibinin-induced apoptosis was prevented by the hydrogen peroxide scavenger catalase and an inhibitor of mitochondrial permeability transition pore, cyclosporine A. These data suggest that ROS generation regulated by a mitochondrial electron transport chain is involved in the silibinin-induced apoptosis.

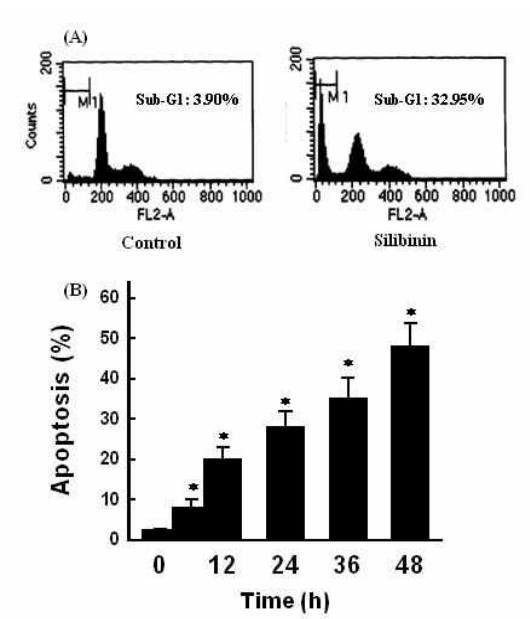

Fig. 1. Effect of silibinin on apoptosis. Cells were exposed to 30 μM silibinin for 36 h (A) and indicated times (B). After exposure, cells were harvested and stained with propidium iodide for determination of apoptotic cells by FACS analysis. Data are mean± SEM of four independent experiments performed in duplicate. *p<0.05 compared with zero time.

3. Effect of silibinin on mitochondrial membrane potential(Ψm)

Since the above data show that silibinin-induced ROS generation is regulated by the mitochondrial electron transport

chain, silibinin may cause disruption of Ψm. Therefore, we examined changes in ym in cells treated with silibinin. Fig. 3A show that the Ψm was decreased after 12 h of silibinin treatment as evidenced by a left shift of the mitochondrial potential sensitive dye $DiOC_6(3)$ fluorescence curves (an increase in M1 gate dregion). This is indicative that silibinin treatment induces collapse of Ψm. Silibinin caused reduction in Ψm after 6 h of treatment and remained unchanged up to 24 h (Fig. 3B). Such changes in Ψm induced by silibinin were prevented by the inhibitors of mitochondrial electron transport chain system (Fig. 3C). The treatment with these inhibitors alone did not significantly affect the Ψm as compared with that in untreated control cells. These data suggest that disruption of Ψm is responsible for the silibinin-induced apoptosis.

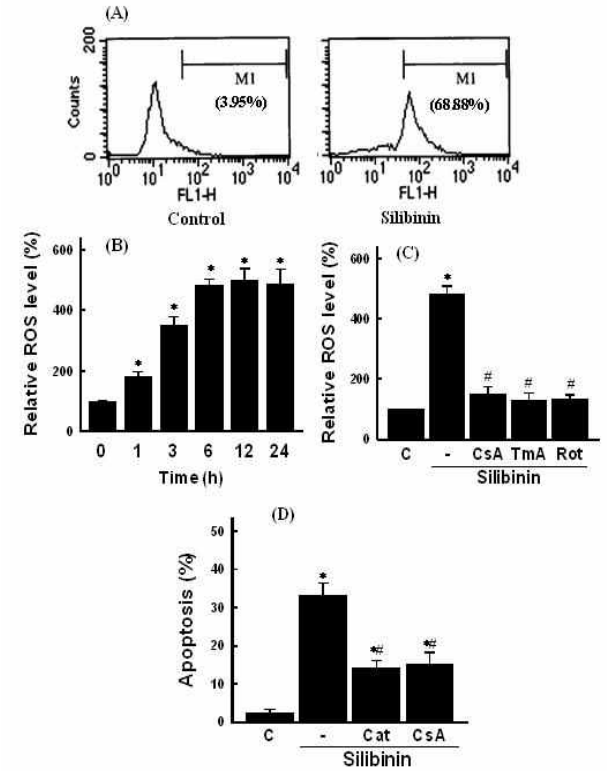

Fig. 2. Role of ROS generation in silibinin-induced apoptosis. Cells were loaded with 30 mM DCFH-DA for 1 h and treated with 30 mM silibinin for 6 h (A) and indicated times (B).Changes in DCF fluorescence was assayed using FACS analysis. Representative (A) and quantitative data (B) of three experiments are shown. Data are mean ± SEM of three experiments performed in duplicate. . *p<0.05 compared with zero time. (C) Effects of inhibitors of mitochondrial electron transport chain on the silibinin-induced ROS generation. Cells were exposed to 30 mM silibinin in the presence or absence of 1 mM cyclosporine A (CsA), 20 mM antimycin A (AmA), and 1 mM rotenone for 6 h and ROS generation was measured as described above. Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control; #p<005 compared with silibinin alone. (D) Effects of the hydrogen peroxide scavenger and mitochondrial electron transport chain inhibitor on silibinin-induced apoptosis. Cells were exposed to 30mM silibinin in the presence or absence of 500 units/ml catalase (Cat) and 1 mM cyclosporine A (CsA) for 36 h and apoptosis was estimated by FACS analysis. Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control; #p<005 compared with silibinin alone.

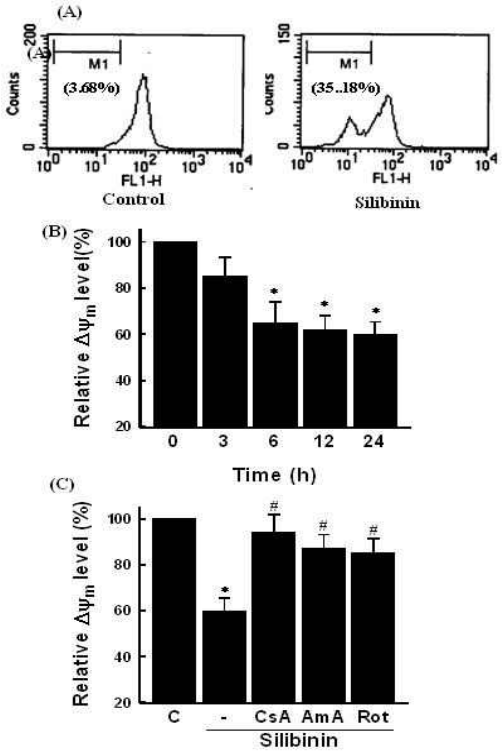

Fig. 3. Effect of silibinin on mitochondrial membrane potential transition (Ψm). Cells were exposed to 30 mM silibinin for 6 h (A) and various times (B). The Ψm was estimated by the uptake of a membrane potential-sensitive fluorescence dye $DiCO_6(3)$. The fluorescence intensity was analyzed using FACS analysis. Data in (B) are mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with control; #p<005 compared with silibinin alone. (C) Effects of inhibitors of mitochondrial electron transport chain on the silibinin-induced disruption of Ψm. Cells were exposed to 30 mM silibinin in the presence or absence of 1 mM cyclosporine A (CsA), 20 mM antimycin A (AmA), and 1 mM rotenone for 6 h and the ym was measured as described above. Data are mean ± SEM of four independent experiments performed in duplicate. * p<0.05 compared with control; # p<005 compared with silibinin alone.

4. Effect of silibinin on cytochrome c release from mitochondria

Disruption in the Ψm induced by silibinin may cause pro-apoptotic proteins such as cytochrome c from mitochondria. To test this possibility, we measured cytochrome c release in cells exposed to silibinin. As shown in Fig. 4A, silibinin caused cytochrome c release after 6 h of treatment. The silibinin-induced cytochrome c release was almost completely inhibited by catalase and cyclosporine A (Fig. 4B).

5. Silibinin induces caspase-dependent cell death

To determine if caspase activation plays a critical role in the silibinin-induced cell death, the effect of silibinin on activation of caspase-3 was examined. Procaspase-3 was decreased after 6 h of silibinin treatment and further decreased up to 24 h (Fig. 5A), indicating activation of caspase-3 by silibinin treatment. Such changes in procaspase-3 expression were prevented by the caspase-3 specific inhibitor DEVD-CHO and the inhibitor of mitochondrial permeability transition pore cyclosporine A (Fig. 5B). To evaluate if caspase-3 activation is

responsible for the silibinin-induced apoptosis, the effect of caspase inhibitors on the cell viability was examined. Cells were exposed to silibinin in the presence of z-DEVD-FMK (general caspase inhibitor) and DEVD-CHO. The silibinin-induced cell death was prevented by these inhibitors (Fig. 5C). These data indicate that silibinin induces apoptosis via a caspase-dependent mechanism.

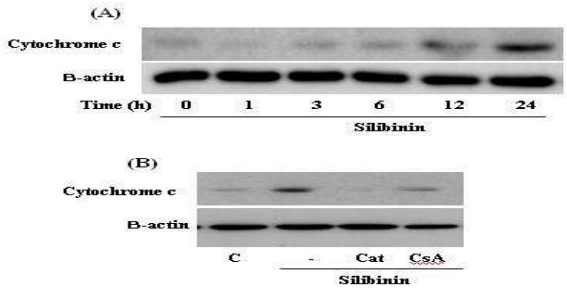

Fig. 4. Effect of silibinin on cytochrome c release. Cells were exposed to 30 μM silibinin for various times (A) and for 24 h in the presence or absence of 500units/ml catalase (Cat) and 1 mM cyclosporine A (CsA) (B). Cytochrome c release into cytosolic fraction was estimated by Western blot analysis. Representative results of two independent experiments are shown.

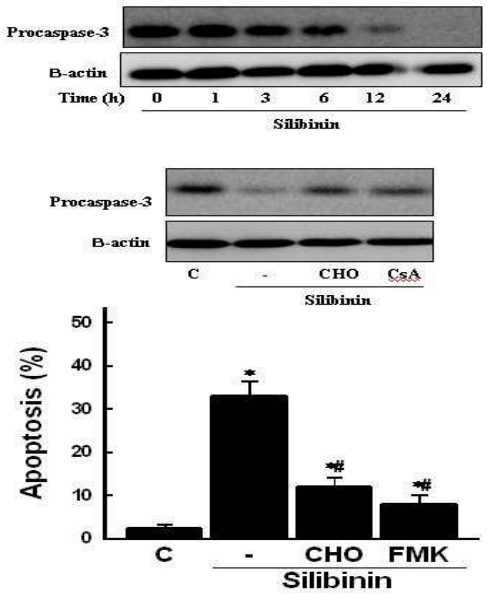

Fig. 5. Role of caspase-3 activation on silibinin-induced apoptosis. Cells were exposed to 30 μM silibinin for various times (A) and for 24 h in the presence or absence of 10 μM DEVD-CHO (CHO) and 1 mM cyclosporine A (CsA) (B). Expression of procaspase-3 was estimated by Western blot analysis. (C) Cells were exposed to 30mM silibinin in the presence or absence of each 10 μM of DEVD-CHO (CHO) and z-VAD-FMK (FMK) for 36 h. Apoptosis was estimated by FACS analysis MTT assay. Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control; #p<005 compared with silibinin alone.

Discussion

Human carcinogenesis is best characterized as an accumulation of alterations in cancer regulating genes such as oncogenes, tumor suppressor, and apoptosis-regulating and DNA-repair genes[17]. In this regard, induction of apoptosis is considered to be one of the important targets in a chemopreventive approach. Over the past few years, it has been shown that flavonoids can trigger apoptosis through the modulation of a number of key elements in cellular signal transduction pathways related to apoptosis[4,18]. The present study demonstrated that silibinin induces apoptosis (Fig. 1).

Although the antioxidant effect of flavonoids has been well known[6], they may also behave as a pro-oxidant, generating ROS, which is responsible for cell death in some cancer cells including glioma cells[19-21]. However, the role of ROS in the silibinin-induced apoptosis is not clear. In the present study, silibinin increased ROS generation and the antioxidant catalase prevented the silibinin-induced apoptosis (Fig. 2). These results indicate that the silibinin-induced apoptosis is attributed to ROS generation.

In the present study, we have demonstrated that silibinin treatment causes ROS generation responsible for the apoptosis. However, it is not known regarding the underlying mechanism by which silibinin causes ROS generation in glioma cells. One possible explanation is that silibinin could directly or indirectly interact with ROS generation systems and causes an increase in ROS generation. However, we have no direct evidence to verify that the increase in ROS generation could be attributed to the silibinin-induced production of free radicals. Another possibility is that silibinin could act on the mitochondrial electron transport system and subsequently cause an increase in ROS generation. Indeed, the inhibition of mitochondrial electron transport chain with antimycin A, rotenone, and cyclosporine A prevented the silibinin-induced ROS generation (Fig. 2C).

Oxidative stress induces the opening of the mitochondrial permeability transition, and subsequently mitochondrial swelling disrupts the outer mitochondrial membrane, leading to release of apoptosis-inducing proteins such as cytochrome c causing the downstream activation of the effector caspases[22]. In the present study, we verified that silibinin induces the mitochondrial dysfunction via the observed elevation of ROS generation, and the reduction in Ψm, accompanied by cytochrome c release from mitochondria into cytosol (Fig. 3 and 4). In addition, inhibition of silibinin-induced mitochondrial disruption with cyclosporine A, a selective inhibitor of Ψm dissipation, prevented the silibinin-induced apoptosis, suggesting that disruption of Ψm plays an important role in the silibinin-induced apoptosis.

The present study showed that the silibinin-induced cytochrome c release was prevented by catalase and

cyclosporine A (Fig. 4B). The silibinin-induced caspase-3 activation also was inhibited by and DEVD-CHO and cyclosporine A (Fig. 5B). These data suggest that the ROS generation and mitochondrial dysfunction were upstream events of cytochrome c release and caspase-3 activation in the silibinin-induced apoptosis.

The mitochondrial apoptotic pathway is initiated by cytochrome c release that promotes the activation of caspase-9 through Apaf-1. The activated caspase-9 then activates the downstream caspase-3[23-25]. The present study shows that silibinin causes mitochondrial dysfunction, cytochrome c release, and caspase-3 activation. These data indicate that silibinin induces apoptosis largely through the mitochondrial pathway.

Taken together, the present study demonstrated that silibinin induces apoptosis through ROS-dependent mitochondrial pathway in human glioma cells. These data suggest that silibinin may be considered a potential candidate in prevention and treatment of human malignant gliomas. However, additional studies for the anticancer efficacy *in vivo* of silibinin are required.

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