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Silibinin induces protective superoxide generation in human breast cancer MCF-7 cells

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Abstract
The pharmacological activity of polyphenolic silibinin from milk thistle (*Silybum marianum*) is primarily due to its antioxidant property. However, this study found that silibinin promoted sustained superoxide ($O_2^{\cdot -}$) production that was specifically scavenged by exogenous superoxide dismutase (SOD) in MCF-7 cells, while the activity of endogenous SOD was not changed by silibinin. Previous work proved that silibinin induced MCF-7 cell apoptosis through mitochondrial pathway and this study further proved that $O_2^{\cdot -}$generation induced by silibinin was also related to mitochondria. It was found that respiratory chain complexes I, II and III were all involved in silibinin-induced $O_2^{\cdot -}$ generation. Moreover, it was found that silibinin-induced $O_2^{\cdot -}$ had protective effect, as exogenous SOD markedly enhanced silibinin-induced apoptosis.

Keywords: *Silibinin, $O_2^{\cdot -}$, SOD, mitochondria*

Introduction

Silibinin (Figure 1) has been clinically used as a liver protective agent. Owing to the polyphenolic structure, amounting evidences implicate that silibinin and its crude form silymarin have antioxidative activity which accounts for the primary mechanism of hepatic protection [1–5]. In recent years, anti-tumour activity of silibinin was also observed in prostate, bladder, hepatoma and lung cancer models [6]. Our previous work found that silibinin induced MCF-7 cell apoptosis through initiating both death receptor and mitochondrial pro-apoptotic pathways [7]. Mitochondrial respiration that usually consumes ~ 90% of the intracellular oxygen is generally considered to be the major generator of cellular reactive oxygen species (ROS) [8], therefore in this study we focus on investigating the role of oxidative stress in modulating MCF-7 cell apoptosis by silibinin. ROS include free radical molecules such as $O_2^{\cdot -}$, hydroxyl free radical ($^{\cdot}OH$) and singlet oxygen ($^{\cdot}O$), as well as non–radical molecules such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) and among them $O_2^{\cdot -}$ is the original form of ROS. Electrons that 'leak' from mitochondrial respiratory chain could reduce O_2 resulting in the generation of $O_2^{\cdot -}$. It is believed that 1–4% of O_2 is reduced in mitochondria by a one–electron reduction to generate $O_2^{\cdot -}$, even during normal oxidative phosphorylation [9]. Within the mitochondrial respiratory chain, $O_2^{\cdot -}$ production primarily occurs by the constant slow transfer of electrons onto O_2 from respiratory chain complex I (NADH:ubiquinone oxidoreductase) and the semiquinone radical located in complex III (ubiquinol:cytochrome *c* oxidoreductase). Complex II participates in both the Krebs cycle and mitochondrial electron transport chain, it oxidizes succinate to provide two electrons to respiratory chain by reducing ubiquinone and there are increasing evidences that complex II is also a predominant

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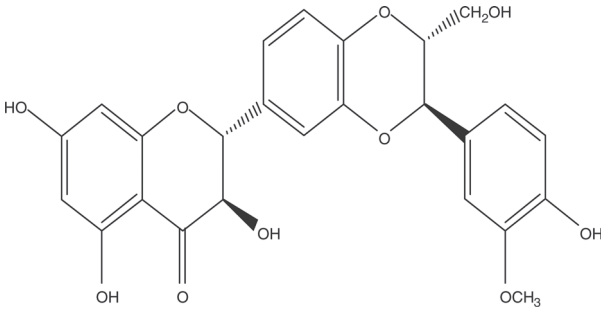

Figure 1. Chemical structure of silibinin.

site for $O_2^{\cdot -}$ generation [10,11]. The function of cytochrome *c* is to transport electrons from complex III to complex IV (cytochrome *c* oxidase), meanwhile cytochrome *c* is also a crucial factor of apoptosome correlating with the initiation of intrinsic apoptosis pathway [12]. ROS were normally considered to be deleterious for its high susceptive reactivity with many biological macromolecules, e.g. proteins, lipids and nucleic acids, but they also occur as potential signalling molecules under sub-toxic conditions [13]. In this work we found that silibinin promoted production of $O_2^{\cdot -}$ and $O_2^{\cdot -}$ scavenging exacerbated silibinin-induced apoptosis in MCF-7 cells.

Materials and methods

Reagents

Silibinin was obtained from the Beijing Institute of Biologic Products (Beijing, China). The purity of silibinin was measured by HPLC and determined to be ~ 99%. Silibinin was dissolved in dimethyl sulphoxide (DMSO) to make a stock solution. The concentration of DMSO was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or death. Crystal violet, propidium iodide (PI), rhodamine-123, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), SOD, N-acetyl-L-cysteine (NAC), catalase, diphenyleneiodonium chloride (DPI), sodium azide (NaN3), rotenone, 2-thenoyltrifluoroacetone (TTFA), stigmatellin, *tert*-butylhydroperoxide (tBHP), glutathione (GSH) and SB203580 were purchased from Sigma Chemical (St. Louis, MO). Enhanced chemiluminescent (ECL) substrate kit was purchased from Pierce Biotechnology (Rockford, IL). Western blotting antibodies against Bcl-x_L, Bcl-2, cytochrome *c*, p38 MAPK, IGF-1Rβ, FAK, EGFR and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Human breast cancer MCF-7 cells were obtained from American Type Culture Collection (#HTB-22™, ATCC, Manasas, VA) and were cultured in MEM medium (Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS) (Dalian Biological Reagent Factory, Dalian, China), L-glutamine (2 mM, Gibco), penicillin (100 μg/ml) and streptomycin (100 ng/ml) at 37°C in 5% CO_2. The cells in the exponential phase of growth were used in experiments.

ROS production measurement

ROS production was monitored by flow cytometry using H2DCFDA as described in another study [14]. H2DCFDA is a stable non-polar compound that readily diffuses into cells and is hydrolysed by intracellular esterase to yield H2DCF, which is trapped within the cells. ROS produced by the cells oxidize H2DCF to the highly fluorescent compound DCF; thus, the fluorescence intensity is proportional to the amount of ROS produced by the cells. The treated cells were incubated with 10 μM H2DCFDA for 30 min at 37°C, then harvested and the pellets were suspended in 1 ml PBS. Samples were analysed with an excitation wavelength at 480 nm and an emission wavelength at 525 nm by a flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Measurement of SOD activity

Total SOD activity was determined with the commercial assay kit (Jiancheng, Nanjing, China). The assay bases on the reaction that SOD inhibits the oxidation of hydroxylamine by the xanthine-xanthine oxidase system [15]. Silibinin-treated cells were washed twice, resuspended in PBS and sonicated for 10s on ice, then centrifuged at 1000 × g for 15 min. The supernatants were subjected to intracellular SOD activity assays. The mauve product (nitrite) produced by the oxidation of hydroxylamine has an absorbance at 550 nm. One unit of the SOD activity was defined as the amount of the sample that reduced the absorbance at 550 nm by 50%.

Western blot analysis

Both adherent and floating MCF-7 cells were harvested, washed twice with ice-cold PBS and then lysed in whole cell lysis buffer (50 μm HEPES, pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF), supplemented with the proteinase inhibitors aprotinin 10 μg/ml, leupeptin 10 μg/ml and pepstatin 100 μg/ml at 4°C for 1 h. After 9500 × g centrifugation at 4°C for 15 min, the protein concentration was determined by a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of total proteins were separated by SDS-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. Proteins were detected

with indicated primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibody and visualized by ECL substrate kit. Preparation of cytosolic extracts was performed as described in our previous work [7].

Flow cytometric analysis of mitochondrial membrane potential

Rhodamine 123 as a cationic fluorescent dye could enter the mitochondrial matrix dependent on mitochondrial transmembrane potential. If mitochondrial membrane potential depleted, the rhodamine 123 could be released from the mitochondria [16]. The mean fluorescence intensity of rhodamine 123 was measured to determine the loss of mitochondrial membrane potential. After incubation with 200 μM silibinin for the indicated time periods, cells were collected and loaded with 5 μM rhodamine 123 for 30 min at 37°C. The fluorescence intensity of cells was analysed by flow cytometry with an excitation wavelength at 488 nm and an emission wavelength at 525 nm.

Growth inhibition assay

The growth inhibitory effect of silibinin on MCF-7 cells was measured by crystal violet staining. The cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) with 1.5×10^4 cells/well density. After 24 h incubation, they were treated with silibinin or other reagents for the indicated time periods. The cells were then washed twice with PBS and stained with 0.5% crystal violet solution containing 20% ethanol at room temperature for 30 min. After washing three times with water, the detained dye was dissolved in 120 μl methanol for each well and the absorbance was measured at 620 nm using an ELISA reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

$$\text{Inhibitory ratio (\%)} = (A_{620,\,\text{control}} - A_{620,\,\text{experiment}})/(A_{620,\,\text{control}} - A_{620,\,\text{blank}}) \times 100$$

Flowcytometric analysis of cell death

MCF-7 cells (1×10^6 cells) were harvested and washed once in cold PBS. The cell pellets were fixed in 75% ethanol at 4°C overnight and washed in cold PBS. Then the pellets were suspended in 1 ml of PI solution containing PI 50 ng/ml, 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X. Cell samples were incubated at 4°C in the dark for at least 15 min and analysed by a flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 630 nm. The percentage of hypodiploid cells with less DNA content than normal diploid ones was counted as death ratio.

Statistical analyses

All data represented at least three independent experiments and were expressed as mean ± SD. The data were analysed by ANOVA using Statistics Package for Social Science (SPSS) software (SPSS, Chicago, IL) and post-hoc test was employed to assess the statistical significance of difference between control and treated groups. *p*-values of less than 0.05 were considered statistically significant.

Results

Silibinin induces ROS generation in MCF-7 cells

The ROS-specific fluorescent probe H2DCFDA was used to investigate silibinin-induced ROS production in MCF-7 cells. The flow cytometric results showed that silibinin induced ROS generation in time- and dose-dependent manners (Figures 2A and B). Compared with 250)im tBHP, a model agent that generated hydroxyl radicals providing a positive control, 200 μM silibinin induced relatively higher fluorescence intensity in MCF-7 cells (Figure 2C).

$O_2^{\cdot -}$ is the dominant ROS induced by silibinin

Exogenous enzyme antioxidants (SOD, catalase) or non-enzyme antioxidants (NAC, GSH) were used to evaluate the ROS emerged in silibinin-treated MCF-7 cells. As shown in Figure 3A, the high amount of ROS (column 1) was apparently scavenged by exogenous SOD (column 2) and NAC (column 3), but not by catalase (column 4) or GSH (column 5). SOD specifically catalyses the reduction of super-oxide anions, thus we speculated that the burst of $O_2^{\cdot -}$contributed to the high level of ROS in silibinin-treated cells. Although SOD activity was increased in silibinin-treated lymphocytes [17], the total SOD activity was not significantly influenced after treatment with silibinin for 24 h ($p > 0.05$ vs control), indicating that ROS generation was not due to change in SOD activity (Figure 3B).

Mitochondria are the major source of $O_2^{\cdot -}$ induced by silibinin

Mitochondrial cytochrome *c*-cytochrome *c* oxidase system plays a pivotal role in antioxidant defence at terminal respiratory chain [18]. Cytochrome *c* oxidizes $O_2^{\cdot -}$, receives electrons from external respiratory chain and is further oxidized by cytochrome *c*

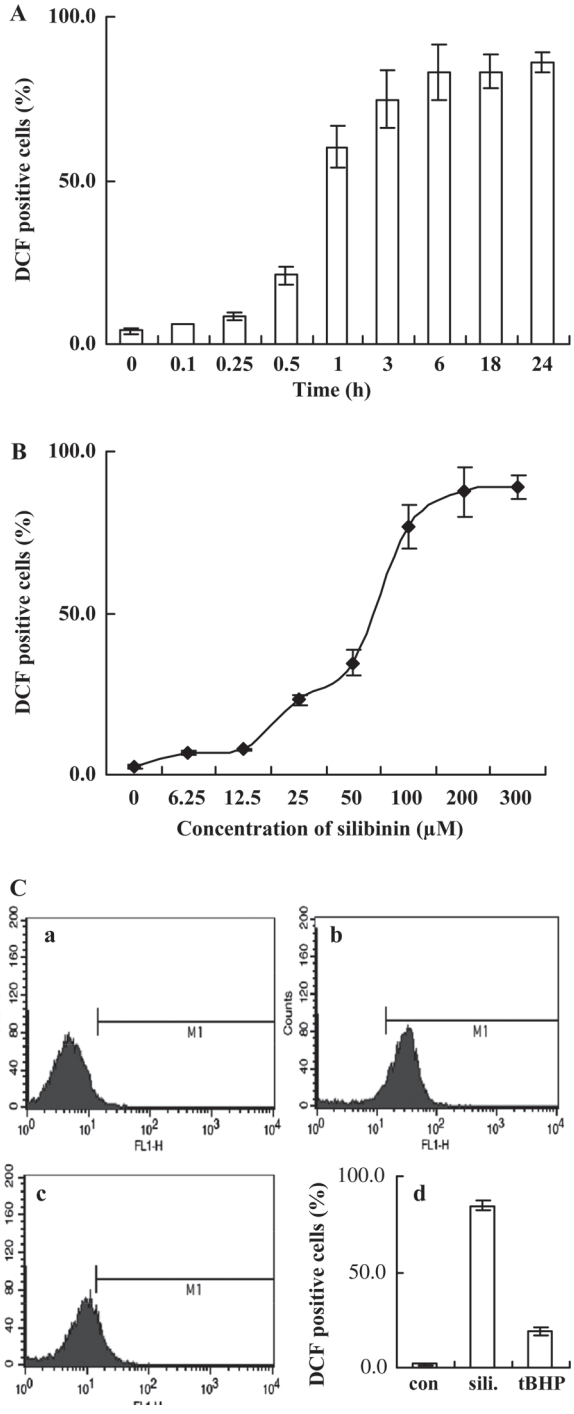

Figure 2. Silibinin induces ROS generation in a time– and dose–dependent manner. MCF-7 cells were treated with 200 um silibinin for 0–24 h (A) or with different concentrations of silibinin for 6 h (B) in MEM media containing 10% FBS and DCF positive cells were detected by flow cytometry. $n = 3$, Mean ± SD. (C) The cells were treated for 24 h with 200 um silibinin or with 250 um tBHP as a positive control. Data from a representative experiment are shown. (a) medium control. (b) silibinin-treated group. (c) tBHP-treated group. (d) The corresponding linear histogram. $n = 3$, Mean ± SD.

oxidase, then transfers the electrons to O_2, forming H_2O. It was demonstrated that DCF positive ratio in silibinin-treated MCF-7 cells was increased 31.9% by 4 mM NaN_3, an inhibitor of cytochrome *c* oxidase

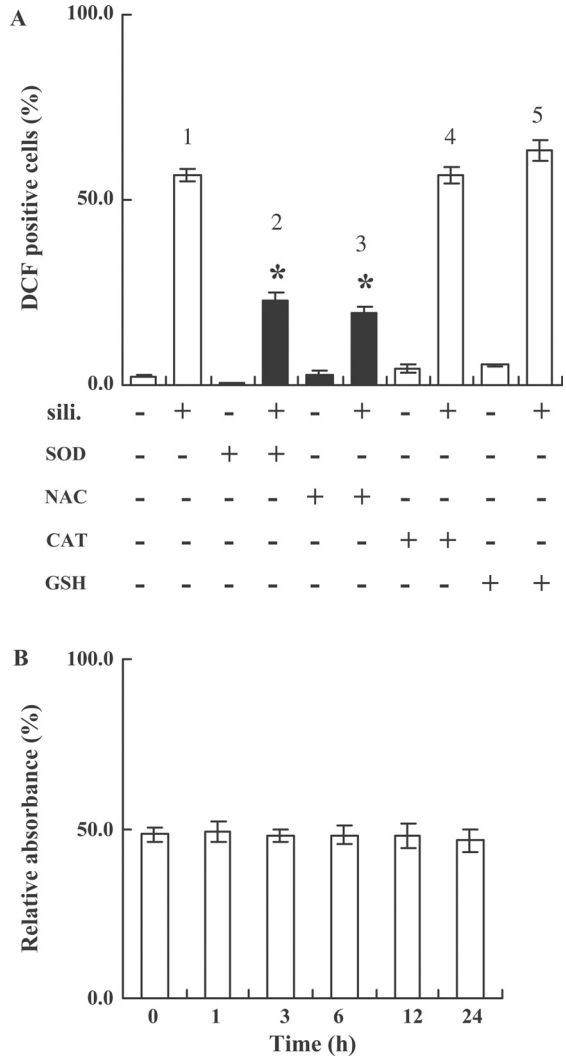

Figure 3. Silibinin induces $O_2^{\cdot -}$ generation without affecting SOD activity. (A) Effect of antioxidants on generation of ROS. MCF-7 cells were treated with 200 um silibinin alone (column 1) for 1 h or pre-treated with 100 U/ml SOD (column 2), 2.5 mM NAC (column 3), 1000 U/ml CAT (column 4) or 2.5 mM GSH (column 5) for 1 h, respectively, and then co–incubated with 200 um silibinin for another 1 h. DCF positive ratio was analysed by flow cytometry. $n = 3$, Mean ± SD. $^*p < 0.01$ vs silibinin alone-treated group. (B) The cells were treated with 200 um silibinin for 0–24 h. The total SOD activity was determined with the assay kit. The samples were diluted to the extent that SOD in the control sample inhibited hydroxylamine oxidation by ~ 50% (48.2% in above results). $n = 3$, Mean + SD.

[19] (Figure 4A). Because NaN_3 did not affect the identified as a main source of $O_2^{\cdot -}$ in the mitochondrial respiratory chain and in the complex I, $O_2^{\cdot -}$ was shown to be produced primarily by the oxidation of reduced flavin or flavin semiquinone [20,21]. Here, compared with silibinin alone-treatment, the DCF positive ratio was decreased 51.3% by addition of a flavinenzyme inhibitor DPI [22] (Figure 4A). We further used different inhibitors of respiratory chain to evaluate the role of complexes I, II and III in silibinin-induced ROS generation (Figure 4B). It was found that the DCF positive ratio was increased

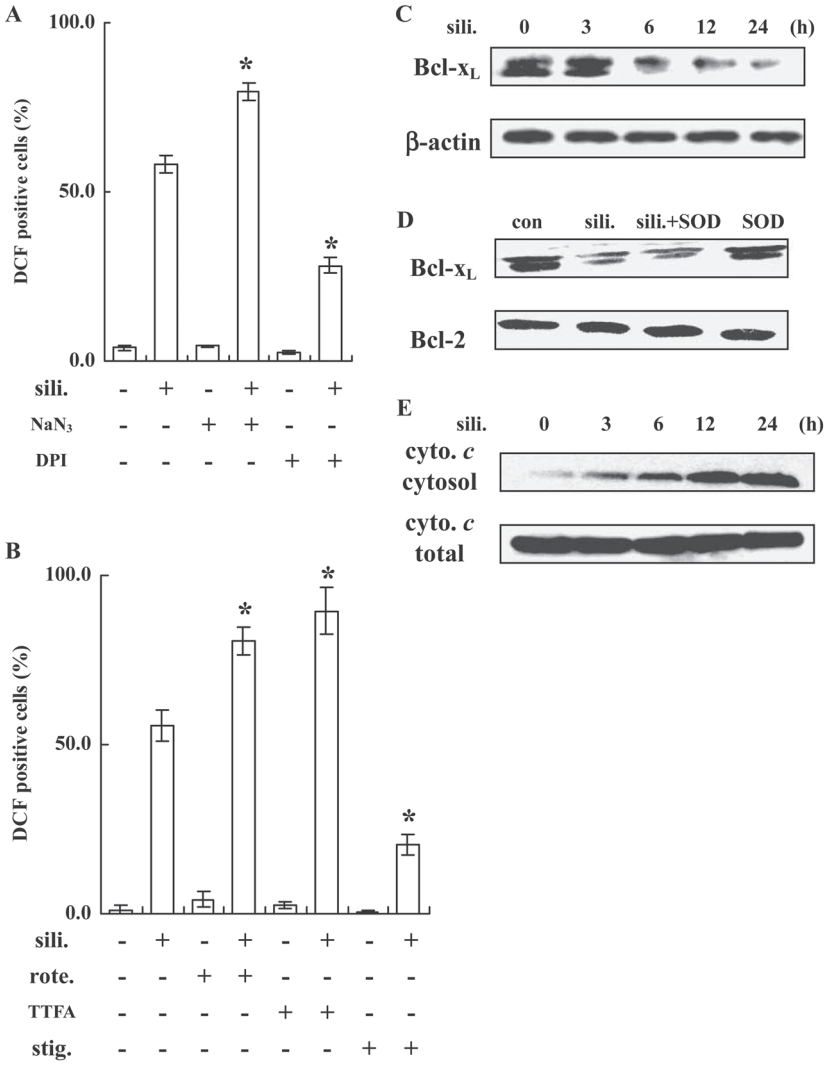

Figure 4. Mitochondrial generation of O2* —. (A) The cells were pre–treated with 20 mMDPIor4 mM NaN_3 for 1 h, respectively, and then coincubated with 200 um silibinin for another 1 h. DCF positive ratio was analysed by flow cytometry. n — 3, Mean ± SD. $^*p < 0.01$ vs silibinin alone-treated group. (B) The cells were pre–treated with 50 um rotenone, 10 um ttfa or 0.1 um stigmatellin for 1 h, respectively, and then co–incubated with 200 um silibinin for another 1 h. DCF positive ratio was analysed by flow cytometry. n —3, Mean + SD. $^*p < 0.01$ vs silibinin alone-treated group. (C) MCF-7 cells were with 200 um silibinin for indicated time periods. The expression of Bcl–xL was examined by Western blot analysis. (D) The cells were pre-treated with 100 U/ml SOD for 1 h and then coincubated with 200 um silibinin for another 12 h. The expression of Bcl-xL and Bcl-2 were examined by Western blot analysis. (E) The cells were pre-treated with 200 um silibinin for indicated time periods. Protein levels of cytochrome *c* in cytosol and whole cell lysate were detected by Western blotting. Data shown are representative of three separate experiments.

$O_2^{\cdot -}$ level in absence of silibinin, we concluded that mitochondrial respiratory chain was the source of silibinin-induced $O_2^{\cdot -}$. Complex I was usually 45.1% by a complex I inhibitor rotenone [23] that blocked electron transfer from complex I to ubiquinone; similarly, TTFA, a complex II inhibitor blocking electron transfer from complex II to ubiquinone by occupying ubiquinone binding sites [24], also significantly promoted silibinin-induced ROS generation and the DCF positive ratio was increased 61.3%. These results demonstrate that silibinin treatment promotes the activation of complex I and II and thus both complex I and complex II were involved in ROS formation in the silibinin treatment cases. Furthermore, stigmatellin, a selective inhibitor that represses $O_2^{\cdot -}$ formation in complex III [25], markedly inhibited ROS generation in silibinin-treated cells and DCF positive ratio was decreased 63.4%. This result demonstrates that complex III is also a site for silibinin-induced ROS generation.

In the circumstances of apoptosis initiation, the anti-apoptotic Bcl-2 family proteins that reside on mitochondrial outer membrane prevent the release of cytochrome *c* from mitochondrial intermembrane space to cytosol where the apoptosome is assembled [26]. We found that the expression of Bcl-x_L was markedly inhibited by silibinin (Figure 4C); moreover, the loss of Bcl-x_L expression was proved to be

not the direct consequence of ROS generation, because SOD did not affect the protein level of Bcl-x_L (Figure 4D), thereby excluding the presumption that Bcl-x_L expression was down-regulated by ROS as reported in the previous study [27]. Accordingly, the level of cytochrome *c* was proved to be increased in cytosolic fraction but not in whole cell lysate (Figure 4E). These results indicated that silibinin promoted the release of cytochrome *c* from mitochondria and interrupted its antioxidant efficacy, leading to the production of ROS.

The dissipation of mitochondrial inner transmembrane potential ($\Delta\Psi$m) is considered to be a general feature of apoptosis [28], nevertheless high $\Delta\Psi$m is also strongly associated with ROS formation [29]. Here silibinin-induced MCF-7 cell apoptosis was accompanied by $O_2^{\cdot-}$ formation, hence we evaluated the changes of $\Delta\Psi$m by the method of rhodamine 123 staining. The result showed that the rhodamine 123 positive ratio had a slight loss of 8.29% only after silibinin treatment for 24 h (Table I), thus the sustained production of $O_2^{\cdot-}$ might be related to relatively normal $\Delta\Psi$m.

ROS scavenging enhances silibinin-induced MCF-7 cell death

Notwithstanding silibinin spurred apoptosis in MCF-7 cells, the concomitant ROS agitation was protective. ROS scavengers SOD and NAC significantly enhanced growth inhibitory effect of silibinin in MCF-7 cells. After treatment for 12 h, the inhibitory ratio was increased from 14.81% to 36.44% and 42.30% by SOD and NAC, respectively; after treatment for 24 h, the inhibitory ratio was further increased from 47.07% to 80.34% and 85.33% by SOD and NAC, respectively (Figure 5A). Apoptotic cells are characterized by DNA fragmentation and consequent loss of nuclear DNA content and the cell death ratio was evaluated by flow cytometric assay of DNA content with PI staining. Silibinin treatment for 12 h induced 6.89% cell death, whereas the death ratio was obviously increased to 15.76% in the presence of SOD; similarly, the death ratio after silibinin treatment for 24 h was increased from 16.82% to 31.83% by addition of SOD (Figure 5B).

Table I. Flow cytometric analysis of mitochondrial $\Delta\psi$m.

Time (h)	Rh 123 positive ratio (%)
0	97.92 ± 1.27
3	96.20 ± 0.15
6	95.90 ± 0.83
12	93.02 ± 1.07
24	89.15 ± 0.43*

The cells were incubated with 200 μM silibinin for 0, 3, 6, 12 and 24 h, then loaded with 5 um fluorescence probe of rhodamine 123. The rhodamine 123 positive ratio was measured by flow cytometry to determine the loss of mitochondrial membrane potential. Values ($n = 3$) are expressed as mean ± SD. $^*p < 0.05$ vs control group.

ROS protects MCF-7 cells by maintaining the expression of tyrosine kinase

Tyrosine kinases such as EGFR, IGF-1R and FAK play critical roles in tumour cell proliferation as well as tumour cell escaping from apoptosis. As shown in Figure 6A, the protein level of EGFR was unexpectedly raised after silibinin treatment, while SOD strikingly diminished both the constitutive and silibinin-induced expression of EGFR. Similar effect of SOD occurred in analysis of IGF-1R and FAK expression, although silibinin alone-treatment did not affect protein level of IGF-1R (Figure 6B). Hence, the protective effect of $O_2^{\cdot-}$ was relevant to regulation of tyrosine kinase expression.

p38 MAPK as a negative feedback target is involved in regulation of $O_2^{\cdot-}$ homeostasis

Mitogen activated protein kinases (MAPKs) such as p38 are important members responding to stress reaction. DCF positive ratio in silibinin-treated cells was decreased from 56.60% to 34.58% by a p38 inhibitor SB203580 [30], giving a sign that p38 contributed to $O_2^{\cdot-}$ formation (Figure 7A). However, the expression of $p38_\beta$ (a p38 isoform of 39 kDa) was obviously inhibited by the augmented $O_2^{\cdot-}$ production, because the down–regulation of p38 was totally reversed by the $O_2^{\cdot-}$ scavenger SOD. Also, it is worth noting that, in contrast to control group, treatment with SOD alone results in much higher expression of $p38_\beta$, as well as $p38_\alpha$ (a p38 isoform of 38 kDa) (Figure 7B). These results demonstrate that p38 is a negative feedback target of both constitutive and silibinin-induced $O_2^{\cdot-}$ and plays a crucial role in maintaining the homeostasis of $O_2^{\cdot-}$ level.

Discussion

As a potential anti-tumour candidate, a phase I clinical trial of silibinin in prostate cancer patients was completed [31]. However, anti-tumour mechanism of silibinin is still needed to be elucidated, as we reported here that cytotoxic dose of silibinin induced protective ROS generation in MCF-7 cells. ROS provoked by silibinin emerges as $O_2^{\cdot-}$, because it is apparently quenched by SOD but not by the H_2O_2 scavenger catalase, $^{\cdot}OH$ scavenger GSH [32,33] or the singlet oxygen quencher NaN3 [34]. $O_2^{\cdot-}$ has a very short half-life under the physiological circumstances, as it is dismutated by mitochondrial MnSOD or cytosolic CuZnSOD giving the formation of H_2O_2. Nevertheless silibinin did not affect the total SOD activity

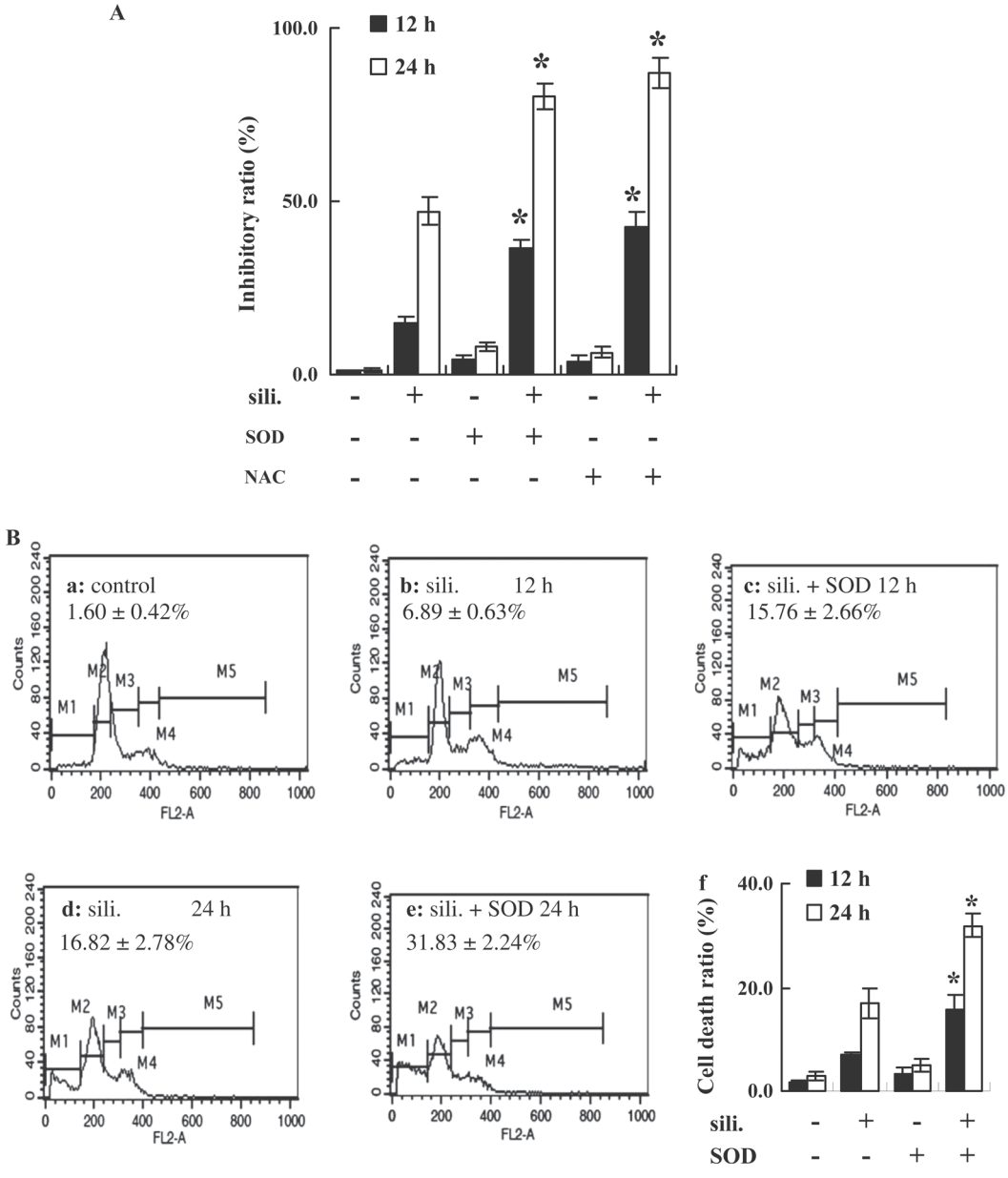

Figure 5. Effect of ROS scavenging on silibinin-induced growth inhibition and death in MCF-7 cells. (A) The cells were pre-treated with 100 U/ml SOD or 2.5 mM NAC for 1 h and then co-incubated with 200 um silibinin for another 12 or 24 h. Growth inhibitory ratio was measured by crystal violet staining. $n = 3$, Mean ± SD. (B) The cells were pre-treated with 100 U/ml SOD for 1 h and then co-incubated with silibinin for another 12 or 24 h, then harvested and stained with PI, followed by flow cytometric detection. The percentage of M1 area cells (hypodiploid cells) is counted as death ratio and the representative flow cytometric histograms with the percentage of M1 area cells on the top are shown. (a) control; (b) silibinin treatment for 12 h; (c) co-treatment with silibinin and SOD for 12 h; (d) silibinin treatment for 24 h; (e) co-treatment with silibinin and SOD for 24 h; (f) the flow cytometric quantification result of dead cells. $n = 3$, Mean + SD. $^{*}p < 0.01$ vs silibinin alone-treated group.

indicating that the high level $O_2^{\cdot -}$ in MCF-7 cells was caused by its aberrant generation. In fact, it has been reported that the antioxidant property of silibinin is due to its potent ability to react with ·OH and silibinin is not an effective scavenger of $O_2^{\cdot -}$ or H_2O_2 [35,36]. ·OH is the most reactive form of ROS and is the major factor that induces oxidative damage. The considerable numbers of reports that silibinin is an antioxidant agent and protects cells from apoptosis might be principally related to the ·OH or lipid peroxide scavenging ability, since scavenge of ·OH and lipid peroxides also prevent the other antioxidant enzyme such as SOD and catalase from damage. Compared with other forms of ROS, $O_2^{\cdot -}$ is more moderate and hardly directlydamages the biomacro molecules. In our research, protective ROS formation is presumed that silibinin induces generation of $O_2^{\cdot -}$ that is a mild form of ROS, while the transformation of $O_2^{\cdot -}$ to a more reactive ROS form such as ·OH is meanwhile prevented by silibinin. We have reported

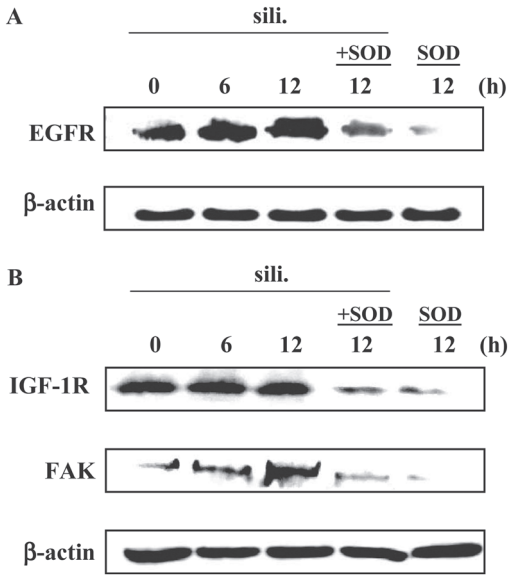

Figure 6. Effect of SOD on expression of tyrosine kinases. (A) The cells were pre-treated with 100 U/ml SOD for 1 h and then co-incubated with 200 μM silibinin for another 12 h. Expression of EGFR was detected by Western blotting. (B) The cells were pre-treated with 100 U/ml SOD for 1 h and then co-incubated with 200 μM silibinin for another 12 h. Protein levels of IGF-1R and FAK were detected by Western blotting. Data shown are representative of three separate experiments.

that silibinin induced MCF-7 cell apoptosis by initiating both the exogenous and mitochondrial apoptotic pathways and this process is correlated with the action of promoting expression of FasL and Bax, as well as Bax translocation to mitochondria and cytochrome *c* release. Therefore, silibinin displays two-way regulating action of promoting apoptosis initiation and protective ROS generation in MCF-7 cells.

Silibinin induces the occurrences of both apoptosis and ROS generation, urging us to focus on mitochondria that play a bifunctional role in apoptosis and oxidative stress. Complexes I and II are the entry points for electrons into the respiratory chain and complex III only funnels electrons from the ubiquinone pool to cytochrome *c*. Complexes I and II oxidize NADH and succinate, respectively, to reduce ubiquinone to ubiquinol. One electron of ubiquinol is transferred to iron-sulphur protein and, subsequently, to cytochrome *c*1 of complex III. The reduced cytochrome *c*1 donates the electron via cytochrome *c* to complex IV. The second electron from ubiquinol flows into the Q-cycle where it is transferred successively to cytochrome *b*L and cytochrome αH. The complex III inhibitor stigmatellin could bind to cytochrome *b* and Rieske iron-sulpher protein, therefore competing with ubiquinol, and prevents electron transfer from ubiquinol to the iron-sulpher centre as well as the reactive semiqui-none formation [25]. In our study, stigmatellin significantly quenched silibinin-induced ROS. Therefore, it can be supposed that silibinin might interfere the bifurcated oxidation of ubiquinol and promote the unstable semiquinone to react with O_2 to form $O_2^{\cdot -}$ at the site of complex III. Other results, that exacerbated ROS generation by interrupting electron transport from complex I and II to ubiquinone with rotenone or TTFA, illustrated that silibinin could also induce complex I and II activation and superfluous electrons generation. Therefore, the overloaded electrons could greatly facilitate $O_2^{\cdot -}$ formation in both in complex I and II and the excess electrons would also flow to complex III where more $O_2^{\cdot -}$ was generated.

It is noticeable that cytochrome *c* is a electron carrier from complex III to complex IV in respiratory chain, as well as a pro-apoptotic factor when released to cytosol. cytochrome *c*, that loosely binds to the outer surface of the inner mitochondrial membrane, can be released to the intermembrane space and operates as a potent enzyme oxidizing $O_2^{\cdot -}$ back to O_2. The reduced cytochrome *c* is oxidized by cytochrome *c* oxidase. Interruption of this process would result in large-scale $O_2^{\cdot -}$ production. Whereas after released to cytosol, cytochrome *c* would bind to cytosolic proteins and then activate caspase-9. Bcl-x_L displays anti-apoptotic action by preventing cytochrome *c* release

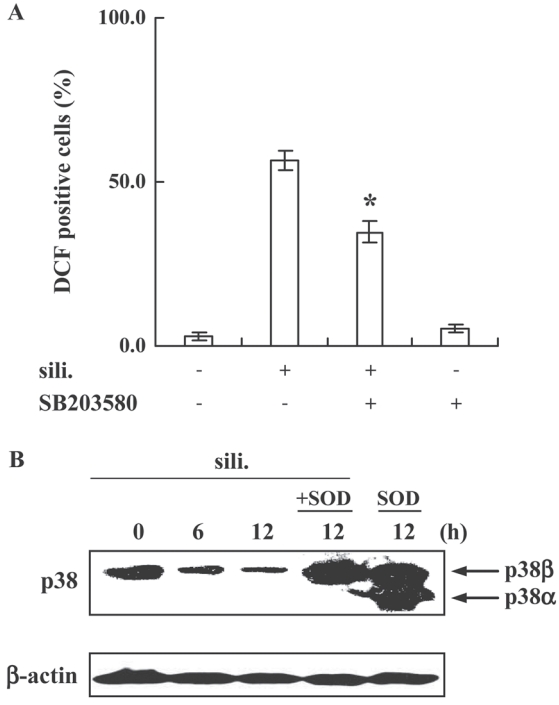

Figure 7. Negative feedback control of p38 MAPK expression by $O_2^{\cdot -}$ (A) MCF-7 cells were pre-treated with 15$^{\cdot -}$ SB203580 for 1 h and then co-incubated with 200 μM silibinin for another 1 h. DCF positive ratio was analysed by flow cytometry. $n=3$, Mean ± SD. $^*p < 0.01$ vs silibinin alone-treated group. (B) In the presence of 100 U/ml SOD, the cells were treated with 200 μM silibinin for 12 h. The protein levels of p38 were examined by Western blot analysis. Repeated three experiments gave similar results.

from mitochondria to cytosol. On the other hand, the formation of Bcl-x_L-cytochrome *c* complex in cytosol also could hamper the initiation of apoptosis [37]. In addition, Bcl-x_L was shown to be more effective in preventing apoptosis than Bcl-2 in MCF-7 cells [38]. Our study found that silibinin significantly depressed Bcl-x_L expression, yet increased Bax expression [7] and facilitated cytochrome *c* release from mitochondria to cytosol. Therefore, both the oxidative burst of $O_2^{\cdot -}$ and the occurrence of apoptosis in MCF-7 cells might be related to cytochrome *c* release induced by silibinin. It was also worth mentioning that $O_2^{\cdot -}$ generation and cytochrome *c* release was not accompanied by $\Delta\Psi$m collapse, because the highest value of $O_2^{\cdot -}$ generation, Bcl-xL depression and cytochrome *c* release all occurred as early as 6 h after silibinin treatment, while the slight loss of $\Delta\Psi$m occurred at 24 h. This manifests that mitochondrial ROS production depends on the existence of high $\Delta\Psi$m and our result is in line with the results of previous studies reporting that $\Delta\Psi$m is requisite for $O_2^{\cdot -}$ production in rat heart or liver mitochondria [29,39]. Hence, one efficacy of silibinin should be augmenting permeability of outer mitochondrial membrane promoting cytochrome *c* release, whilst maintaining the relative integrity of inner membrane $\Delta\Psi$m.

EGFR, IGF–1R and FAK are crucial regulators in uncontrolled tumour cell growth and migration, as they cross talk each other or activate the common downstream kinases such as MAPK and Akt. FAK is essential for integrin-stimulated cell migration [40]. The FERM domain of FAK facilitates a signalling linkage from EGFR [41] and FAK also interacts with IGF–1R to promote survival [42]. EGFR and FAK signalling were shown to be inhibited by silymarin and silibinin [43,44]. However, we found that expression of EGFR and FAK was markedly upregulated by silibinin in MCF-7 cells and this process was eliminated by $O_2^{\cdot -}$ scavenging. It has been reported that exogenous $O_2^{\cdot -}$ can stimulate growth in hamster fibroblasts (BHK-21), Balb/3T3 cells, human amnion cells, mouse epidermal cells (JB6) and human histiocytic leukaemia cells (U937) [45]. In these cell lines, $O_2^{\cdot -}$ or H_2O_2 was found to stimulate the expression of early growth-related genes such as c-fos and c-jun and the growth stimulating effect of $O_2^{\cdot -}$ was extremely rapid, thus leading to the suggestion that $O_2^{\cdot -}$ might function as mitogenic stimuli through the growth factor receptor pathway. Besides, NF-*k*B is also a well known redox–regulated transcription factor [46,47]. It has been reported that EGFR expression and cell proliferation were induced via NF-*k*B and activator protein-1 (AP-1) pathways [48], therefore $O_2^{\cdot -}$ might induce EGFR expression by regulating the activity of correlative transcription factors. Some other reports also implicate the mechanism that $O_2^{\cdot -}$ induces tyrosine kinase expression [49–51].

In our other work, it was found that a tyrosine kinase inhibitor genistein, an IGF-1R inhibitor AG1024 [7] or an EGFR inhibitor AG1478 notably promoted silibinin-induced MCF-7 cell apoptosis. Here we found silibinin-induced cell death was apparently enhanced by addition of SOD, therefore we further investigated the effect of $O_2^{\cdot -}$ scavenging on the expression of tyrosine kinases. In our experiments, 100 U/ml SOD alone treatment did not affect the viability of MCF-7 cells. Whereas SOD alone treatment at this concentration was found to apparently inhibit the protein levels of EGFR, IGF–1R and FAK and this action existed at 12 h when silibinin alone treatment induced marginal apoptosis. However, combined treatment with SOD obviously enhanced silibinin-induced apoptosis at this time point. The results indicate that silibinin induces amplification of a series of apoptotic signals [7] as well as the down-regulation of anti-apoptotic signal such as Bcl-x_L, however, this progress is also accompanied by tyrosine kinase signal enhancement; in contrast, $O_2^{\cdot -}$ scavenger SOD suppresses the protein level of tyrosine kinases, but not the level of the anti-apoptotic protein Bcl–x_L and thus it can be supposed that SOD promotes silibinin-induced cell death via down–regulating tyrosine kinase expression. Although silibinin had no obvious effect on IGF-1R expression, ROS quenching promoted the degradation of IGF-1R, implying that $O_2^{\cdot -}$ might be involved in post-transcriptional regulation of IGF-1R.

MAPKs are mediators of signalling from receptor tyrosine kinases to the nucleus, however molecular mechanisms of MAPKs such as p38 are still not well established [52,53]. It was reported that p38 can be activated by mitochondrial ROS [54], but it was not clear whether and how p38 regulated ROS generation. We found that p38 participated in $O_2^{\cdot -}$ promotion in silibinin-treated MCF-7 cells, whereas the elevated $O_2^{\cdot -}$ level meanwhile repressed the expression of p38. Therefore, $O_2^{\cdot -}$ generation is p38 independent considering notable inhibition of p38 expression by silibinin, but p38 is indeed a potent regulator that maintains $O_2^{\cdot -}$ in a homeostatic level.

In summary, silibinin-induced MCF-7 cell apoptosis is in concomitance with ROS producing from mitochondria and this process is further augmented by cytochrome *c* release and well regulated by ROS-p38 reverse feedback circuit. However the propagation of $O_2^{\cdot -}$ can also be harnessed by tumour cells to avoid apoptosis, thus our findings cast the enlightenment that redox–based signalling should be validated as a promising target for silibinin to prevent tumours.

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