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Silibinin induces the generation of nitric oxide in human breast cancer MCF-7 cells

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Abstract
Increasing research has concentrated on the anti-tumour efficacy of silibinin, a flavonolignan that is clinically used as an hepatoprotectant. However, previous work has found that silibinin-induced apoptosis is accompanied by protective superoxide (O_2•-) generation in MCF-7 cells. This study further reports the formation of reactive nitrogen species (RNS) in the same system. It finds that silibinin induces nitric oxide (•NO) generation in a time- and concentration-dependent manner. Moreover, the results support that there exists an inter-regulation pattern between RNS and reactive oxygen species (ROS) generation. In addition, silibinin is also found to induce RNS and ROS generation in the isolated populations of mouse peripheral blood mononuclear cells (PBMCs) and a simple *in vivo* model of *Caenorhabditis elegans*.

Keywords: *Silibinin, •NO, ROS*

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Introduction

Silibinin, a flavonolignan from the seeds and fruits of milk thistle (*Silybum marianum*), is used in the clinic or as dietary supplements against liver toxicity in Asia, Europe and the US [1]. Antioxidant activity is an important mechanism for silibinin, preventing damage of hydroxyl free radical (•OH) and lipoperoxide that are supposed to play critical roles in hepatotoxicity and other pathogenesis of inflammation, ischaemia/reperfusion, atherosclerosis and ageing. However, our previous research prove that silibinin induces protective O_2•– generation in the MCF-7 cell line and the mitochondrial respiratory chain (MRC) complexes I, II and III are involved in O_2•$^-$ formation [2]. Here we further find that O_2•$^-$ production is concomitant with •NO generation.

•NO is synthesized by nitric oxide synthases (NOS) using L-arginine and molecular oxygen. The family of NOS is normally categorized into three isozymes: the neuronal (nNOS, type I), the endothelial (eNOS, type III) and the inducible (iNOS, type II) isoforms, nevertheless the expressions of these isoenzymes are not tissue-specific [3]. •NO is a weak oxidant which interacts with a limited number of biomolecules, in particular those containing heme moieties, and reversibly modifies the function of different mitochondrial enzymes acting as a physiological modulator of mitochondrial respiration [4]. However, •NO produced in aerobic environments can turn to a potent oxidant of peroxynitrite ($ONOO^-$) by reacting with O_2•$^-$. Mitochondria are energy producing organelles, as well as necessary links of intracellular signal transduction, e.g. both of silibinin-initiated apoptosis and O_2•$^-$ generation in MCF-7 cells need mitochondria-based network [2,5]. •NO gas, due to its high affinity to cytochrome *c* oxidase (MRC complex IV), diffuses freely from cytosol to mitochondria, binds to complex IV and elicits a burst of O_2•$^-$ and H_2O_2 that

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subsequently diffuse outside mitochondria and accumulates in cytosol or nucleus [6]. In contrast, $ONOO^-$ has little or no effect on complex IV but inhibits complexes I, II and III [7]. On the other hand, ROS such as $O_2^{\bullet -}$ directly or indirectly activates redox-sensitive transcriptional factors such as NF-κB and AP-1 that are involved in expression of many pro-inflammatory genes including iNOS [8,9].

Oxidative stress involving RNS and ROS have both pro- and anti-apoptotic effects and the different levels of oxidative stress in distinct milieu would direct diverse signalling pathways to cell proliferation, differentiation, apoptosis and senescence [10]. Excessive RNS/ROS leads to protein nitrosation or oxidation, DNA damage, apoptosis and necrotic cell death [11]. Some anti-apoptotic effects of RNS/ROS include inhibition of caspases by S-nitrosylation/oxidation and induction of cytoprotective gene expression such as HSPs, Bcl-2 and Akt/PKB [12]. Furthermore, redox status is clearly related to the activity of growth factors and to cell transformation and cacinogenesis [13]. In this work, we find oxidative stress induced by silibinin at a cytotoxic concentration is protective and is augmented by a way of inter-regulation between RNS/ROS.

Materials and methods

All animals use procedures were in accordance with the Regulation of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. The experiments were carried out under the approval of the Committee of Experimental Animal Administration of the University.

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. 4,5 Diaminofluorescein diacetate (DAF-2DA), 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), sodium nitroprusside (SNP), N-(3-(Aminomethyl)benzyl)acetamidine (1400w), iron(III) *meso*-tetra(2,4,6-trimethyl-3,5- disulphonato) porphine chloride (FeTMPS), diphenyleneiodonium chloride (DPI), superoxide dismutase (SOD), N-acetyl-L-cysteine (NAC), stigmatellin, DL-Dithiothreitol (DTT), catalase, tyrphostin AG1024, tyrphostin AG1478, genistein, wortmannin, SP600125, SB203580, manumycin A, GW5074, PD98059 and crystal violet were purchased from Sigma Chemical (St. Louis, MO). Enhanced chemiluminescent (ECL) substrate kit was purchased from Pierce Biotechnology (Rockford, IL). Western blotting antibodies against iNOS, c-Jun N-terminal kinase (JNK1/2), ERK, phosphorylated ERK, Ras, caspase 8 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 (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 U/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37°C. The cells in the exponential phase of growth were used in experiments.

Animals and diets

Female Swiss-Kunming mice, weighing between 20–25 g, were used in the experiments, which were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). They were housed four per cage, maintained at 22 ± 0.5°C with a controlled 12 h light-dark cycle and with *ad libitum* water and food.

PBMC preparations

Venous blood from Swiss-Kunming mice was collected in heparinized tubes and centrifuged over a Ficoll-Hypaque cushion (Sigma). PBMCs were collected from the Ficoll/Hypaque interphase and washed three times in MEM medium and then cultured in MEM medium with 10% FBS for 24 h prior to further treatment with silibinin.

C. elegans culture and treatment

The wildtype *C. elegans*, Bristol N2, were obtained from the *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN). The worm stocks were cultured on bacterial lawns of *E. coli* strain OP50 (obtained from the CGC) on standard nematode growth media (NGM) agar plates at 20°C according to standard methods [14]. Experimental cultures were set up by transferring eggs from NGM agar plates into S-media [14] using a sterile platinum wire. Young adults were treated with 200 μM silibinin for 24 h and then stained with 10 μM DAF-2DA or 10 μM H2DCFDA at 20°C for 30 min. The animals were anaesthetized in 0.5 mM levamisole and images were collected with a fluorescence microscope (Olympus, Tokyo).

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Excitation and emission wavelengths were 485 and 515 nm for DAF-2T and 480 and 525 nm for DCF.

RNS production measurement

The intracellular RNS is detected using DAF-2DA that is membrane permeant and is deacetylated by intracellular esterases to 4,5-diaminofluorescein (DAF-2). DAF-2 is essentially non-fluorescent until it is nitrosylated by products of oxidation of nitric oxide to form fluorescent DAF-2 triazole (DAF-2T), which become trapped in the cytoplasm. The treated cells were collected and resuspended in PBS and then incubated with 10 μM DAF-2DA at 37°C for 30 min, then harvested and the pellets were suspended in 1 ml PBS. Samples were analysed with an excitation wavelength at 485 nm and an emission wavelength at 515 nm by a flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Other methods

ROS production measurement, growth inhibition assay by crystal violet staining and Western blot analysis were performed as described in our previous work [2].

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 •NO/ONOO⁻ generation in MCF-7 cells

The generation of •NO was detected by flow cytometry with •NO-specific fluorescent probe DAF-2DA. The DAF-2T positive ratio in MCF-7 cells was increased by silibinin treatment in a time- and concentration-dependent manner (Figures 1A and B). After treatment for 6 h, the DAF-2T positive ratio was increased to 61.16% and 11.23%, respectively, by 200 μM silibinin and 250 μM SNP (a •NO donor) (Figure 1C). The DAF-2T positive ratio was remarkably decreased by a selective iNOS inhibitor 1400w [15] (Figure 1D). Furthermore, the Western blot result demonstrated that iNOS expression was apparently elevated by silibinin (Figure 1E). However, silibinin-induced DAF-2T positive ratio was also obviously decreased by an $ONOO^-$ scavenger FeTMPS. Another result demonstrated that DAF-2T positive ratio was markedly decreased by DPI, both an NOS inhibitor [16] and a MRC complex I inhibitor that had been found to inhibit ROS formation in silibinin-treated MCF-7 cells [2] (Figure 1D). Thus, silibinin induces co-generation of $O_2^{\bullet -}$ and •NO in MCF-7 cells, as well as $ONOO^-$ since it can be formed by the diffusion-limited reaction between •NO and $O_2^{\bullet -}$ [17,18].

Inter-regulation between RNS and ROS generation

Both RNS and ROS are induced by silibinin in MCF-7 cells, which urges us to investigate the potential inter-relation between ROS and RNS generation. In order to clearly reflect that whether different reagents promote or inhibit the production of •NO (Figure 2A) and ROS (Figure 2C), we choose the time ranges when 50~60% MCF-7 cells are •NO positive (silibinin treatment for 6 h) or ROS positive (silibinin treatment for 1 h). Our results demonstrated that DAF-2T positive cells induced by silibinin significantly reduced in number after co-treatment with exogenous $O_2^{\bullet -}$ scavenger SOD or non-selective antioxidant NAC, as well as stigmatellin, a MRC complex III inhibitor, but not by catalase (Figure 2A). Although there is a possibility that the above antioxidants might inhibit the $ONOO^-$ formation and accordingly decrease the DAF-2T positive ratio, the following Western blot analysis proved that SOD treatment obviously reversed the up-regulated iNOS expression by silibinin (Figure 2B). It is worth noting that DAF-2T positive cells are totally eliminated by a •NO scavenger DTT at 500 μM (Figure 2A). Although DTT is also a scavenger of ROS and $ONOO^-$, 23.4% and 31.7% cells are still ROS positive after treatment with 500 μM DTT for 1 h (Figure 2C) and 6 h (data not shown), respectively. These results suggest that synthesis of ROS is •NO-independent. On the other hand, we used ROS-specific fluorescent probe H2DCFDA to investigate ROS level in the presence of RNS scavengers. The results demonstrated that silibinin-induced ROS was significantly depressed by the iNOS inhibitor 1400w (Figure 2C), while significantly increased by the •NO donor SNP. Moreover, DCF positive ratio is increased by FeTMPS implying that $ONOO^-$ negatively regulates ROS formation.

Inhibition of IGF-1R and JNK promotes silibinin-induced RNS/ROS generation

In our previous work, both receptor tyrosine kinases (RTKs) and $O_2^{\bullet -}$ were proved to antagonize silibinin-induced apoptosis in MCF-7 cells, therefore it should be necessary to investigate the roles of RTKs in regulating RNS/ROS generation. The results demonstrated that silibinin-induced RNS/ROS formation

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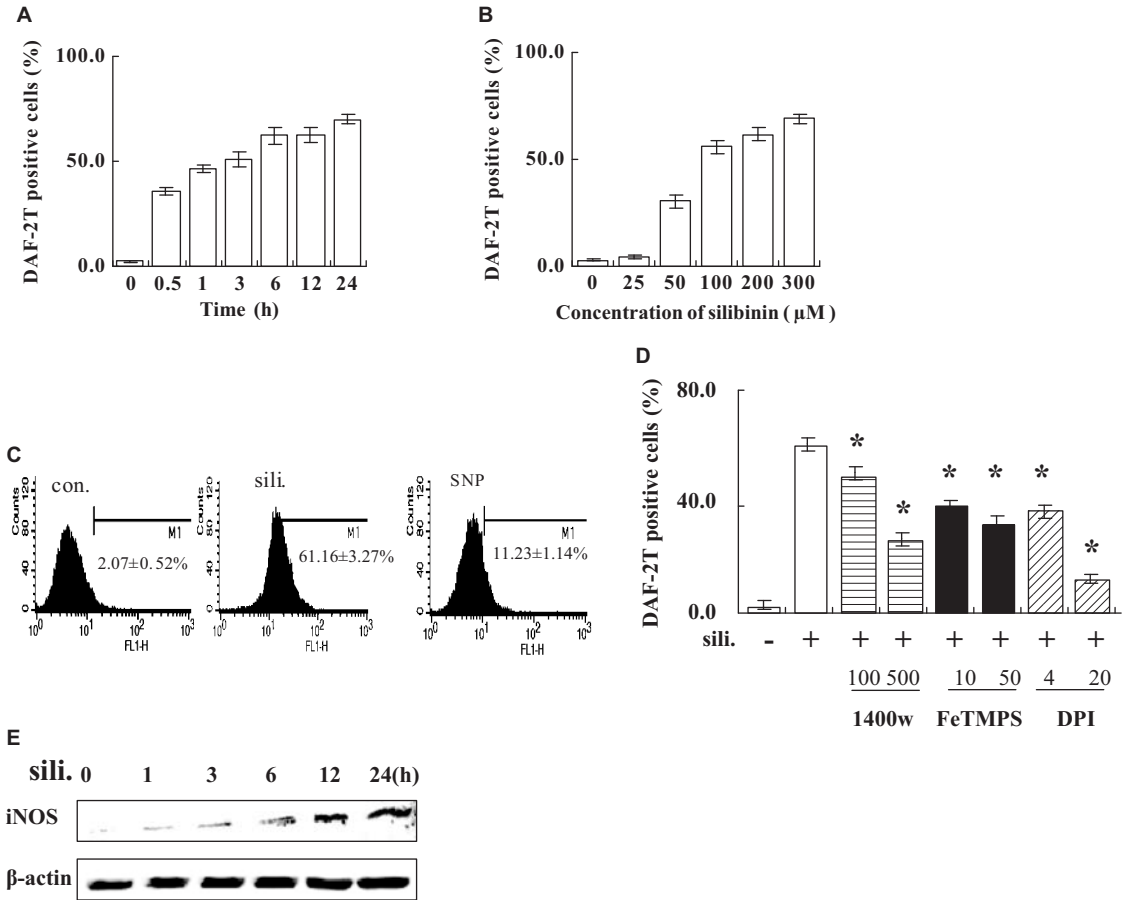

Figure 1. Silibinin induces RNS generation in a time- and concentration-dependent manner. MCF-7 cells were treated with 200 μM silibinin for 0–24 h (A) or with different concentrations of silibinin for 6 h (B), DAF-2T positive cells were detected by flow cytometry, $n=3$, Mean ± SD. (C) The cells were treated for 6 h with 200 μM silibinin or with 250 μM SNP as a positive control. Vertical and horizontal axis values represent cell number and the intensity of DAF-2T fluorescence, respectively. Data from a representative experiment are shown, $n=3$, Mean ± SD. (D) MCF-7 cells were treated with 200 μM silibinin alone for 6 h or the cells were pre-treated with 100 or 500 μM 1400w, 10 or 50 μM FeTMPS or 4 or 20 mM DPI for 1 h, respectively, and then coincubated with 200 μM silibinin for another 6 h. DAF-2T positive ratio was analysed by flow cytometry, $n=3$, Mean ± SD, $^{*}p < 0.01$ vs silibinin alone-treated group. (E) MCF-7 cells were treated with 200 μM silibinin for indicated time periods. The expression of iNOS was examined by Western blot analysis. Data shown are representative of three separate experiments.

was markedly enhanced by an insulin-like growth factor 1 receptor (IGF-1R) inhibitor AG1024, a general tyrosine kinase inhibitor genistein or serum deprivation but not by an epidermal growth factor receptor (EGFR) inhibitor AG1478 (Figures 3A and B). Next, we investigated the effects of the classical downstream members of RTK pathway on RNS/ROS formation. It was found that a JNK inhibitor SP600125 strongly enhanced silibinin-induced RNS/ROS production. Whereas RNS positive cells were significantly decreased by a Ras inhibitor manumycin A and a p38 MAPK inhibitor SB203580 and slightly decreased by a PI3K inhibitor wortmannin, a Raf-1 inhibitor GW5074 or an ERK inhibitor PD98059 (Figure 3C) and ROS positive cells were significantly decreased by PD98059 (Figure 3D) and SB203580 that had been reported in Wang et al. [2]. The subsequent Western blot analysis illustrated that the expression of JNK was obviously depressed by silibinin and this depression was not affected in the presence of SOD or 1400w (Figures 3E and F), suggesting that silibinin down-regulated JNK expression in a redox status-independent way. We have reported that silibinin inhibits the expressions of p38 [2] and other results demonstrate that silibinin decreases the phosphorylation level of ERK and does not affect Ras expression (Figures 3G and H). Thus, IGF-1R and JNK might be involved in negative regulation of RNS/ROS formation.

RNS/ROS antagonize silibinin-induced cytotoxicity

Compared with silibinin alone treatment, increased cytotoxicity by co-treatment with 1400w and silibinin was moderate. In addition, co-treatment with FeTMPS did not affect the cytotoxicity of silibinin (Figure 4A). Nevertheless, co-treatment with exogenous catalase or with DTT, the scavenger of both RNS and ROS, significantly promoted silibinin-induced cytotoxicity (Figure 4A). It was found that

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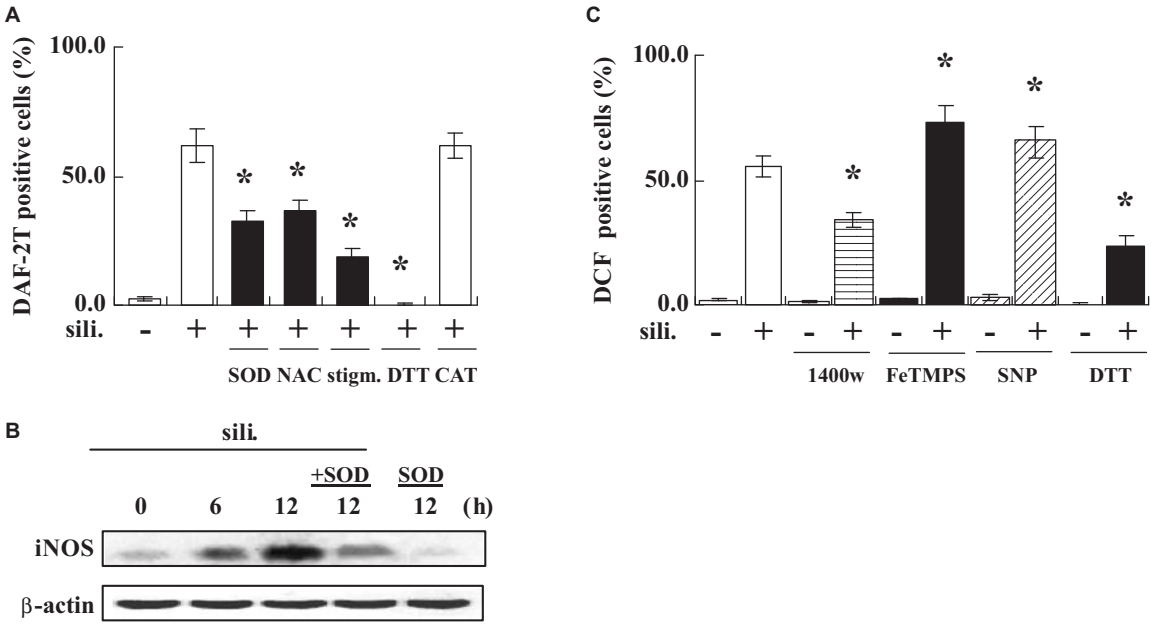

Figure 2. The inter-regulation between RNS/ROS generation. (A) MCF-7 cells were pre-treated with 100 U/ml SOD, 2.5 mM NAC, 0.1 μM stigmatellin, 500 μM DTT or 1000 U/ml catalase (CAT) for 1 h and then coincubated with 200 μM silibinin for another 6 h. DAF-2T positive ratio was analysed by flow cytometry, $n=3$, Mean±SD, $^*p < 0.01$ vs silibinin alone-treated group. (B) MCF-7 cells were pre-treated with 100 U/ml SOD for 1 h and then coincubated with 200 μM silibinin for another 12 h. Protein levels of iNOS were detected by Western blotting. Data shown are representative of three separate experiments. (C) MCF-7 cells were treated with 200 μM silibinin alone for 1 h or the cells were pre-treated with 500 μM 1400w, 10 μM FeTMPS, 250 μM SNP or 500 μM DTT for 1 h and then coincubated 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.

exogenous catalase apparently promoted silibinin-induced activation of caspase 8 (Figure 4B). Therefore, in this system •NO/$ONOO^-$ mainly participate in promoting and maintaining ROS level, while $O_2•^-$ and H_2O_2 perform the apoptosis antagonist.

Silibinin induces RNS/ROS generation in mouse PBMCs and C. elegans

To investigate whether silibinin-induced RNS/ROS generation was specific for MCF-7 cell line, the isolated mouse PBMCs and *C. elegans* were subjected to silibinin treatment. It was found that after treatment with silibinin for 6 and 18 h, respectively, 20.18% and 25.59% PBMCs were DAF-2T positive and 20.23% and 26.46% PBMCs were DCF positive (Figure 5A). It was also found that apparent DAF-2T and DCF fluorescence was induced in *C. elegans* by treatment with silibinin (Figure 5B). These results demonstrate that silibinin also induces ROS/RNS generation in PBMCs and *C. elegans*.

Discussion

RNS and ROS are major components of the intracellular signalling pathway and the production of RNS/ROS would interrupt the efficacy of chemotherapeutics by influencing the redox status [19,20]. For example, ROS at physiological conditions control the activity of transcriptional factors such as NF-κB and AP-1 which regulate the expression of cell growth mediators [12,21]. However, the over-produced ROS would lead to uncontrolled activities of transcriptional factors which promote abnormal cell proliferation [13,22]. We have found that silibinin induced $O_2•^-$ generation in MCF-7 cells and $O_2•^-$ subsequently antagonizes silibinin-induced apoptosis by promoting the expression of EGFR, IGF-1R and FAK. Here we further report that silibinin-induced $O_2•^-$ promotes iNOS expression and •NO generation and these results agree with the report that SOD significantly suppressed LPS-induced iNOS expression and •NO production [23]. In contrast, silibinin-induced •NO also plays an important role in regulating ROS formation. DAF-2DA is generally utilized as a fluorescent detector for •NO in cells and tissues, the product derived from •NO auto-oxidation nitrosates DAF-2 to yield the highly fluorescent DAF-2T. It was reported that $ONOO^-$ or $O_2•^-$ alone did not induce DAF-2T fluorescence, whereas •NO-induced DAF-2T fluorescence would be increased in the presence of $ONOO^-$ [24]. Although it is quantitatively difficult to determine the ratio of •NO to $ONOO^-$ under condition that $O_2•^-$ and •NO are co-generated, our data support that both •NO and $ONOO^-$ are major forms of RNS induced by silibinin treatment. It is well known that •NO inhibits complex IV [25–27] and interrupts its antioxidant function, while $ONOO^-$ selectively inhibits respiratory complexes I–III [7]. This mechanism is supported by

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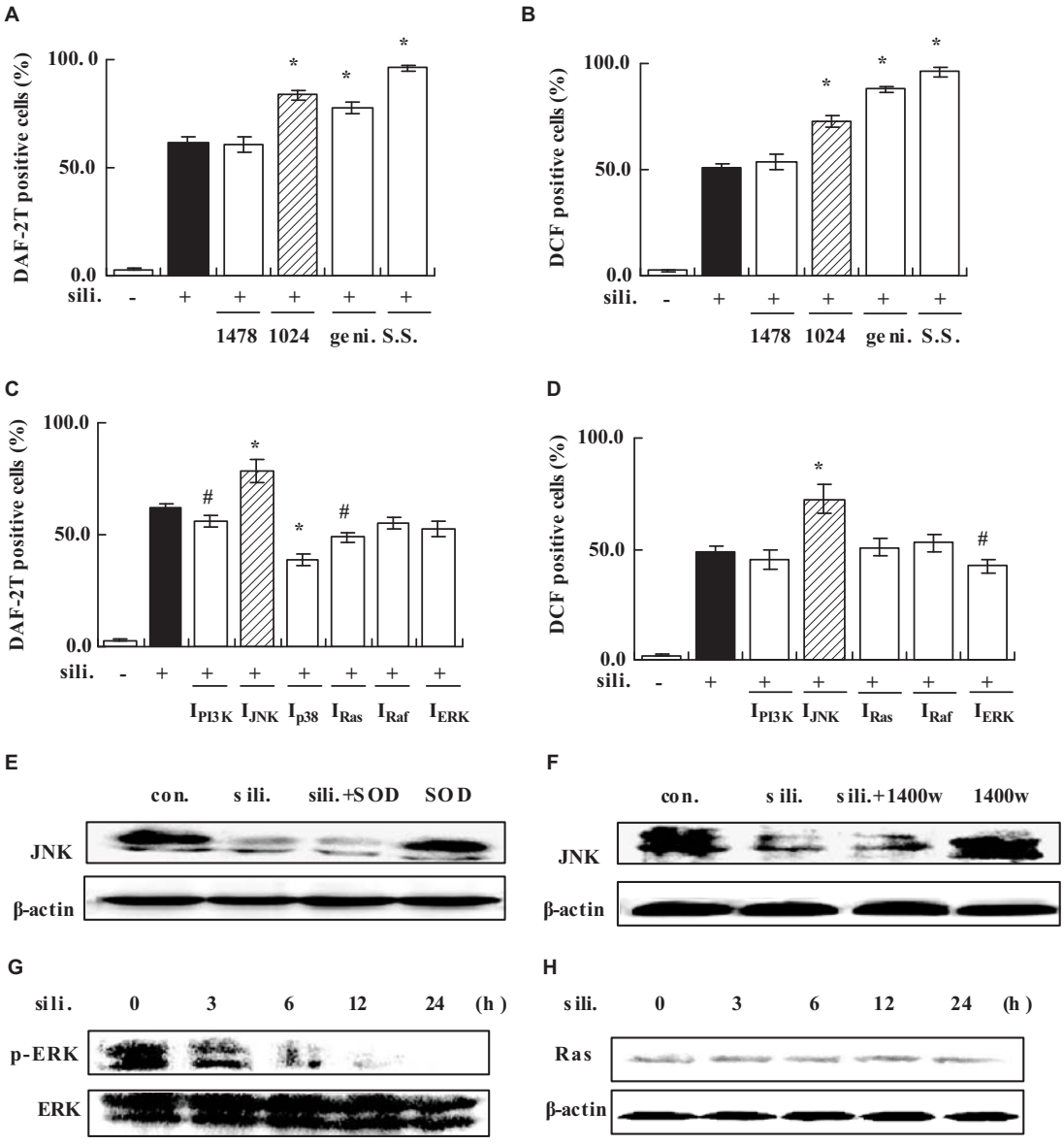

Figure 3. Effects of the members of RTK signalling pathway on RNS/ROS generation. MCF-7 cells were treated with 200 μM silibinin alone for 6 h (A) or 1 h (B) or the cells were pre-treated with 12 μM AG1478, 4 μM AG1024, 50 μM genistein (geni.) or serum starvation (S. S.) for 1 h, respectively, and then coincubated with 200 μM silibinin for another 6 h (A) or 1 h (B). DAF-2T (A) or DCF (B) positive ratio was analysed by flow cytometry. (C, D) MCF-7 cells were treated with 200 μM silibinin alone for 6 h (C) or 1 h (D) or the cells were pre-treated with 300 nM wortmannin (I_{PI3K}), 20 μM SP600125 (I_{JNK}), 15 μM SB203580 (I_{p38}), 20 μM manumycin A (I_{Ras}), 40 μM GW5074 (I_{Raf}) or 20 μM PD98059 (I_{ERK}), respectively, and then coincubated with 200 μM silibinin for another 6 h (C) or 1 h (D). DAF-2T (C) or DCF (D) positive ratio was analysed by flow cytometry. Compared with control group, treatment alone with different inhibitors or SS did not apparently affect RNS/ROS levels (data not shown), $n = 3$, Mean ± SD, $*p < 0.01$ vs silibinin alone-treated group, $\# p < 0.05$ vs silibinin alone-treated group. (E, F) The cells were pre-treated with 100 U/ml SOD (E) or 500 μM 1400w (F) for 1 h and then coincubated with 200 μM silibinin for another 12 h. Protein levels of JNK were detected by Western blotting. Data shown are representative of three separate experiments. (G, H) MCF-7 cells were treated with 200 μM silibinin for indicated time periods. The phosphorylation level of ERK (G) and the expression of Ras (H) were examined by Western blot analysis. Data shown are representative of three separate experiments.

our results that ROS formation is decreased by inhibition of iNOS, while facilitated by $ONOO^-$ scavenging, because we have reported that a complex IV inhibitor NaN_3 promoted ROS formation in silibinin-treated MCF-7 cells and complexes I–III were all associated with silibinin-induced ROS generation.

Intracellular signalling including ROS/RNS signals is a complex network, the generation of ROS/RNS is well regulated by this network and the regulating process would greatly affect the action of silibinin on tumour cells. We have found that silibinin had no effect on IGF-1R expression, whereas scavenging $O_2\bullet^-$ by SOD decreased the protein level of IGF-1R implying $O_2\bullet^-$ played a key role in maintaining IGF-1R at a steady state. Here IGF-1R is found to be a negative regulator of ROS formation, further

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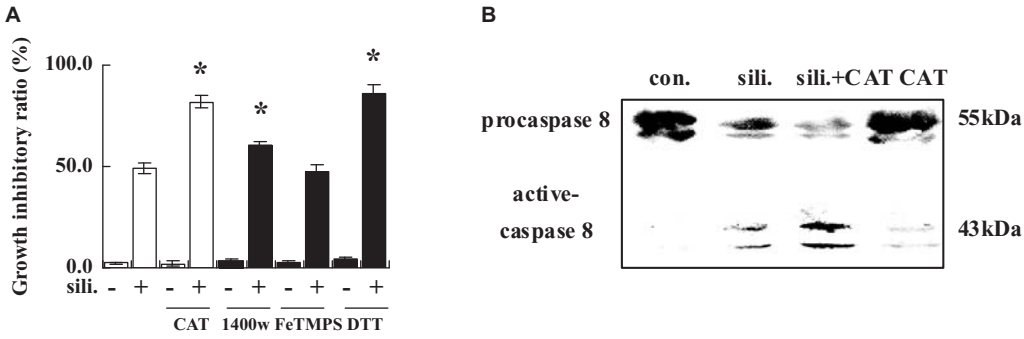

Figure 4. The effects of RNS/ROS scavengers on silibinin-induced cytotoxicity. (A) MCF-7 cells were treated with 200 μM silibinin alone for 24 h or the cells were pre-treated with 1000 U/ml CAT, 500 μM 1400w, 50 μM FeTMPS or 500 μM DTT for 1 h and then coincubated with 200 μM silibinin for 24 h. Growth inhibitory ratio was measured by crystal violet staining, $n=3$, Mean ± SD, $^{*}p < 0.01$ vs silibinin alone-treated group. (B) MCF-7 cells were treated with 200 μM silibinin alone for 24 h or the cells were pre-treated with 1000 U/ml CAT for 1 h and then coincubated with 200 μM silibinin for 24 h. The protein levels of caspase 8 were detected by Western blotting. Data shown are representative of three separate experiments.

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suggesting that a negative feedback loop of $O_2^{\bullet -}$ IGF-1R should be established. This result agrees with our early report that the IGF-1R inhibitor AG1024 promotes dissipation of cytochrome *c* [5], which is a member of the cytochrome *c*-complex IV antioxidant system at terminal MRC. JNK expression was intensively down-regulated by silibinin, whereas a JNK inhibitor SP600125 still critically promoted silibinin-induced RNS/ROS. This phenomenon might be attributed to silibinin's partial inhibition of JNK expression/activity and the elucidation of potential mechanism still needs further study. There is no evidence to link JNK activation and mitochondrial $O_2^{\bullet -}$ formation; however, the elevated ROS level by the JNK inhibitor SP600125 in this experimental system hints that JNK inhibits silibinin-induced mitochondrial malfunction.

On the base of our data that $O_2^{\bullet -}$ is the major form of ROS and promotes survival in silibinin-treated MCF-7 cells, here we further evaluated the roles of H_2O_2 and •NO in cell survival. We have found endogenous SOD activity was not affected by silibinin and it might be reasonable to presume that endogenous SOD would catalyse the dismutation of $O_2^{\bullet -}$ into H_2O_2, whereas ROS level was not significantly affected by exogenous catalase [2]. This phenomenon suggests that intracellular H_2O_2 should be maintained at a relatively low level by endogenous catalase in MCF-7 cells. However, it was found that exogenous catalase notably enhanced silibinin-induced cytotoxicity and our work

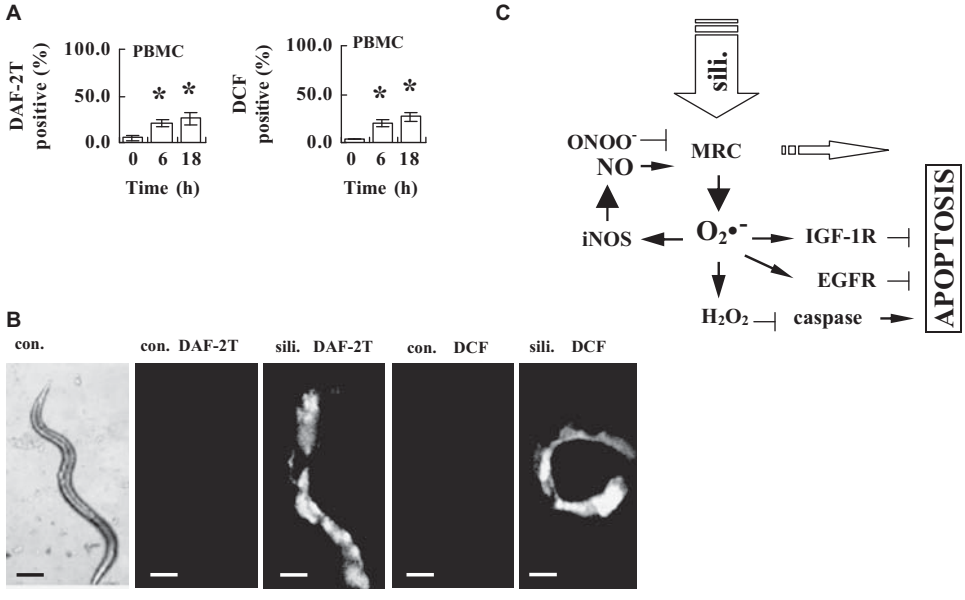

Figure 5. Silibinin induces RNS/ROS generation in mouse PBMCs and *C. elegans*. (A) The DAF-2T or DCF positive ratio were detected by flow cytometry in isolated mouse PBMCs after treatment with 200 μM silibinin for 6 h and 18 h, $n=3$, Mean ± SD, $^{*}p < 0.01$ vs control group. (B) The *C. elegans* were incubated with 200 μM silibinin for 24 h and then observed by a phase contrast microscope (leftmost panel; scale bar, 100 μm) or by a fluorescence microscope after DAF-2DA or H2DCFDA staining (middle and right panels; scale bar, 30 μm). (C) Model of silibinin-induced RNS/ROS generation in MCF-7 cells. MRC, mitochondrial respiratory chain.

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demonstrated that catalase apparently facilitated silibinin-induced activation of caspase 8. Compared with O_2•$^-$ and H_2O_2, •NO plays a minor role in regulating MCF-7 survival in the case of silibinin treatment that might be due to its nature of weak oxidant. Here we also found exogenous catalase did not affect •NO level, indicating O_2•$^-$ but not H_2O_2 was involved in the inter-regulation of RNS and ROS generation.

In conclusion, silibinin induces MCF-7 cell apoptosis in concomitance with RNS/ROS formation (Figure 5C). Inter-regulation of O_2•$^-$ and •NO generation leads to the further augmentation of oxidative stress, while $ONOO^-$ serves as negative feedback regulators to facilitate the homeostasis of O_2•$^-$. The protective action of O_2•$^-$ is primarily due to its maintaining the expression of RTKs such as IGF-1R and EGFR, while H_2O_2 antagonizes silibinin-induced apoptosis by preventing caspase activation. Thus, this study also supports that the strategy of oxidative stress scavenging is very valuable for improving the anti-tumour effect of silibinin in MCF-7 cells. Silibinin induces RNS/ROS generation in various species from nematode to isolated mouse tissue cells, suggesting this mechanism is not specific for the MCF-7 cell line. Together, our research elucidates the generating mechanism and apoptosis antagonizing mechanism for silibinin-induced RNS/ROS and it is a valuable supplement to the character of silibinin being both an antioxidative hepatoprotectant and an anti-tumour candidate.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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