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Mechanism of autophagy induction and role of autophagy in antagonizing mitomycin C-induced cell apoptosis in silibinin treated human melanoma A375-S2 cells

Yuan-yuan Jiang [a], Ri Yang [a], Hong-jun Wang [a], Huai Huang [a], Di Wu [a], Shin-ichi Tashiro [b], Satoshi Onodera [b], Takashi Ikejima [a,*]

[a] China–Japan Research Institute of Medical Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China
[b] Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194-8543, Japan

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ABSTRACT

The aim of this study was to elucidate the molecular mechanisms mediating silibinin-induced autophagy in A375-S2 cells. In the present study it was found that silibinin-induced autophagy through increasing the conversion of LC3 I to LC3 II and up-regulating Beclin-1 expression, which was concomitant with p53 suppression and NF-κB activation. P53 inhibitor, pifithrin-α (PFT-α), increased autophagy and enhanced the expression of NF-κB. Moreover, inducing p53 accumulation with MG132 reduced autophagic ratio, and repressed the expression and activation of NF-κB expression. NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) suppressed autophagy. Autophagic specific inhibitor 3-methyladenine (3-MA) treatment reversed silibinin-induced p53 suppression as well as NF-κB activation, suggesting that there was a positive feedback loop between p53 inhibition-mediated NF-κB activation and autophagy. In addition, we also found that 3-MA efficiently abrogated silibinin's cyto-protective effect against mitomycin C-induced cell death, and reversed the suppressive efficacy of silibinin on p53 expression, suggesting that autophagy contributed to silibinin's cyto-protective effect against mitomycin C-induced cell death in A375-S2 cells.

1. Introduction

Macroautophagy (hereafter autophagy), first discovered in mammalian cells in 1960s, is a highly conserved process in eukaryotic cells. It orchestrates cells self-digesting their own long-lived proteins, organelles or DNA, underscoring its critical role in cellular homeostasis (Terman et al., 2007). The autophagic process is robustly up-regulated in response to cellular stress, such as nutrient or cytokine depletion, hypoxia and oxidative damage, and it is also pivotal to innate intracellular defense mechanism against certain pathogens (Meredith et al., 2010). Besides, autophagy is also induced in the processes of many anti-cancer therapies, and is considered to be a major, tumor cell intrinsic, resistance mechanism (Chen et al., 2010). Thus, autophagy is essential in modulating cell homeostasis, death and survival.

Some critical proteins that are directly involved in autophagic process initiation and formation, such as Atg5, Atg 6, Atg 8 and Atg 12, have been well established (Suzuki and Ohsumi, 2007). However, autophagic process is also modulated by other proteins and other signaling pathways. For instance, in HeLa cells, the activation of death receptor CD95 mediated JNK activation dependent autophagy (Zhang et al., 2008); and in mouse fibroblast sarcoma L929 cells, ERK- and JNK-MAPKs were involved in TNF-α-induced autophagy (Cheng et al., 2008), suggesting that the induction and regulation of autophagy were quite complicated and probably cell specific.

Silibinin (Fig. 1A) is a flavonoid compound abstracted from seeds of *Silybum marianum (L.) Gaertn.* It has multi-pharmacological effects in the treatment of liver and gallbladder disorders, including hepatitis and cirrhosis, and has anti-cancer efficacies such as anti-prostate cancer and anti-bladder cancer (Singh et al., 2003; Davis-Searles et al., 2005). Besides, silibinin is also applied in clinic or as dietary supplements against liver toxicity in Asia, Europe and the United States for many years (Wang et al., 2010). However, the role of silibinin in regulating autophagy, and the molecular mechanisms are still unknown. Our previous study reported that silibinin antagonized mitomycin C-induced intrinsic apoptosis via suppressing p53 expression (Jiang et al., 2009). And at the same study system, silibinin-induced autophagy was also found by us. As we previously described that autophagy could exist as a cyto-protective mechanism in a certain context, thus in the present study we investigated whether and through which mechanism that suppression of p53 was correlated with autophagy induction, and we also elucidated the role of autophagy in silibinin antagonizing mitomycin C-induced apoptosis.

2. Materials and methods

2.1. Materials

Silibinin was obtained from the China Institute of Biological Products (Beijing, China). 5-diphenyl tetrazolium bromide (MTT), propidium

* Corresponding author. Fax: +86 24 2384 4463.
E-mail addresses: emma_840101@hotmail.com (Y. Jiang), ikejimat@vip.sina.com (T. Ikejima).

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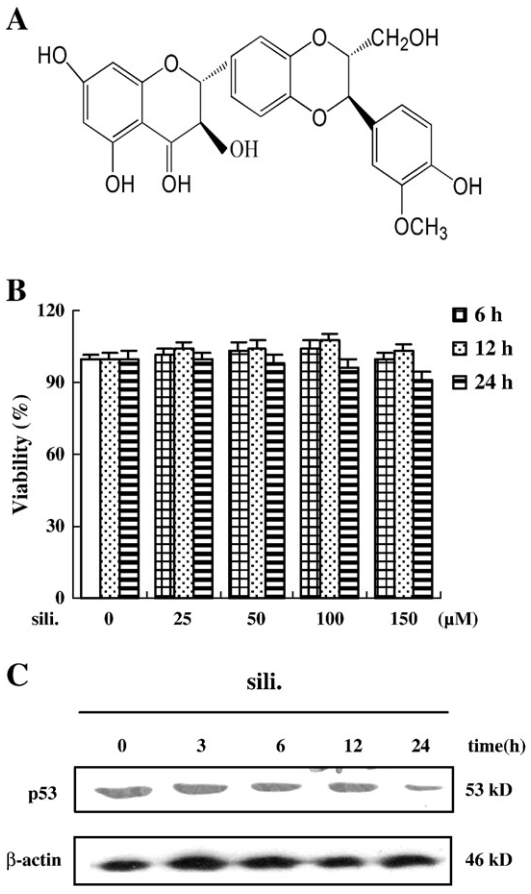

Fig. 1. Silibinin down-regulated the protein levels of p53 in A375-S2 cells. A) The chemical structure of silibinin. B) The cells were treated with 0, 25, 50, 100 and 150 μM silibinin (sili.) for 0, 6, 12 and 24 h and cell viability was analyzed with MTT assay. C) The cells were treated with silibinin at 150 μM for 0, 3, 6, 12 and 24 h before collection and lysis. The expression of p53 was detected by Western blot analysis.

iodide (PI), lipopolysaccharide (LPS), monodansylcadaverine (MDC) and 3-methyladenine (3-MA) were from Sigma Chemical (St. Louis, MO, USA). Rabbit polyclonal antibodies against p53, β-actin, Beclin 1, LC3, NF-κB, p-NF-κB, I-κBα, p-I-κBα, horseradish peroxidase-conjugated secondary antibodies, p53 inhibitor pifithrin-α (PFT-α), proteasome inhibitor MG132, and NF-κB inhibitor Pyrrolidine dithiocarbamate (PDTC) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrochemiluminescence (ECL) was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

2.2. Cell culture

The human melanoma A375-S2 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

2.3. Cytotoxicity assay

A375-S2 cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 0.8×10^4 cells per well and cultured for 24 h. Thereafter the cells were treated with various concentrations of silibinin or mitomycin C for indicated time periods or the cells were treated with 3-MA, PFT-α, PDTC for 1 h prior to silibinin treatment for 24 h. After that the cells were rinsed with ice-cold PBS twice and incubated with 5 mg/L MTT solution at 37 °C for 2 h. The resulting crystal was dissolved in 150 μl DMSO and the optical density was measured by MTT assay using a plate microreader (TECAN SPECTRA, Wetzlar, Germany). Cell viability was calculated as follows (Wang et al., 2008):

$$\text{Cell viability}(\%) = 100 \times (\text{A492, sample} - \text{A492, blank}) \div (\text{A492, control} - \text{A492, blank})$$

2.4. Flow cytometric analysis of autophagy

A375-S2 cells (0.8×10^6/flask) were inoculated in 25 ml culture flasks and cultured for 24 h. The cells were treated with silibinin for 0, 6 12 and 24 h, or the cells were pre-treated with 3-MA, PFT-α, PDTC or MG132 for 1 h and co-incubated with silibinin for 24 h, or the cells were treated with or without PDTC for 1 h and incubated with LPS for 24 h. The collected cells were suspended in 0.05 mM autophagy vacuole specific dye MDC at 37 °C for 1 h (Kabeya et al., 2000). Then cells were analyzed with flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the emission wavelength at 525 nm. The fluorescent intensity of intracellular MDC reflected the number of autophagic cells (autophagic ratio).

2.5. Fluorescent microscopy of autophagy with (MDC) staining

A375-S2 cells (2.8×10^5/well) were inoculated in 6-well culture plates and cultured for 24 h. The cells were treated with or without silibinin for 24 h prior to 0.05 mM MDC incubation at 37 °C for 1 h. Then the fluorescent changes were observed by Olympus IX70 reverse fluorescence microscopy (Olympus, Tokyo, Japan) with the emission wavelength at 525 nm (Zhang et al., 2008).

2.6. Flow cytometric analysis of apoptosis with PI staining

PI was a fluorescent dye that can specifically bind with DNA. The cells were treated with and without 3-MA prior to mitomycin C and silibinin co-treatment for 12 h. The collected cells were fixed with 500 μl PBS and 10 ml 70% (v/v) ethanol at 4 °C over night. Then the cells were rinsed with ice cold PBS twice and suspended with 1 ml PI solution (PI 50 mg/l and RNase A 1 g/l) at a dark place for 15 min. Then the samples were analyzed by FACScan flow cytometer (Kabeya et al., 2000).

2.7. Western blot analysis

Both adherent and floating cells were collected and lysed with protein lysis buffer. Then the cells were centrifuged at $12{,}000\times g$ for 10 min, and the protein content of the supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). The proteins were separated with by 12% SDS-polyacrylamide gel electrophoresis and blotted onto wet nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). And the protein bands were visualized by using anti-rabbit Ig G conjugated with peroxidase, DAB and ECL as described previously (Jiang et al., 2009).

2.8. Statistical analysis

All data represented at least three independent experiments and were expressed as mean ± S.D. The data were analyzed by ANOVA using Statistics Package for Social Science (SPSS) software (SPSS Inc., Chicago, IL, USA). P values <0.05 were considered to be statistically significant.

3. Results

3.1. Silibinin time-dependently suppressed p53 expression in A375-S2 cells

The cells were treated with silibinin at indicated concentrations, and the cell viability was measured by MTT assay. As shown in Fig. 1B, no obvious growth inhibition was found in cells treated with silibinin at a concentration range from 0 to 150 μM. We chose silibinin at the concentration of 150 μM as used in our previous study to conduct our subsequent study. As shown in Fig. 1C, silibinin at the concentration of 150 μM time-dependently suppressed p53 expression below basic cellular level as measured by Western blot analysis.

3.2. Silibinin time-dependently induced autophagy in A375-S2 cells

The cells were treated with silibinin for 0, 6 12 and 24 h in the presence or absence of autophagic specific inhibitor 3-MA. Then the autophagic ratios were measured by flow cytometric analysis of MDC staining as described in Materials and methods. As shown in Fig. 2A, treatment of the cells with silibinin increased autophagic ratio in a time-course manner, and the autophagic ratio was decreased by autophagy inhibitor 3-MA. In the cells treated with silibinin for 24 h, the intense punctuate MDC fluorescence, which represented the autophagic vacuoles, was clearly observed by fluorescent microscopy of MDC staining (Fig. 2B). As shown in Fig. 2C, there was a only slight decrease in cell viability in 3-MA and silibinin co-treated group (93.47 ± 2.25%) compared to that of silibinin treated alone group (89.77 ± 1.02%, $p = 0.137$), and no statistical significance was found between the two groups.

3.3. Autophagy was under negative control of p53 in silibinin treated A375-S2 cells

Since p53 suppression and autophagy induction occurred simultaneously in silibinin treated cells, we next focused on studying whether there was any crosstalk between p53 and autophagy. The cells were pre-treated with p53 inhibitor PFT-α for 1 h and then co-incubated with silibinin for 24 h. As shown in Fig. 3A, co-treatment of the cells with silibinin and p53 inhibitor PFT-α resulted in an evident rise in autophagic ratio as determined by flow cytometric analysis of MDC staining (37.08 ± 1.94% with PFT-α and silibinin co-treated group vs. 23.08 ± 2.03% with silibinin treated group, $p = 0.00024$). The protein level of autophagy-associated protein Beclin 1 (mammalian homologue of Atg 6) and the conversion of LC3 I to LC3 II (a biological marker for autophagy vacuole formation) were assessed by Western blot analysis to further investigate autophagy induction in the cells co-treated with PFT-α and silibinin. Result from Western blot analysis showed that, in the cells co-treated with silibinin and PFT-α, there was prominent increase in the expression of Beclin 1 and in the conversion of LC3 I to LC3 II (Fig. 3B, left panel). As shown in the right panel of Fig. 3B, the increased protein densitometric ratio of LC3 I/LC3 II was further augmented by PFT-α pre-treatment (3.73 ± 0.13 with PFT-α and silibinin treated group vs. 2.62 ± 0.12 with silibinin treated alone group, $p = 0.016$). Conversely, treatment of the cells with proteasome inhibitor MG132, which blocked the degradation of p53 protein by proteasome, increased the protein levels of p53 (Fig. 3C), and decreased the number of autophagic cells (Fig. 3A) (17.08 ± 1.06% with MG132 and silibinin treated group vs. 23.08 ± 2.13% with silibinin treated alone group, $p = 0.0030$). Moreover, result from Western blot analysis revealed that the up-regulation of Beclin 1 protein and the conversion of LC3 I to LC3 II were reversed by MG132

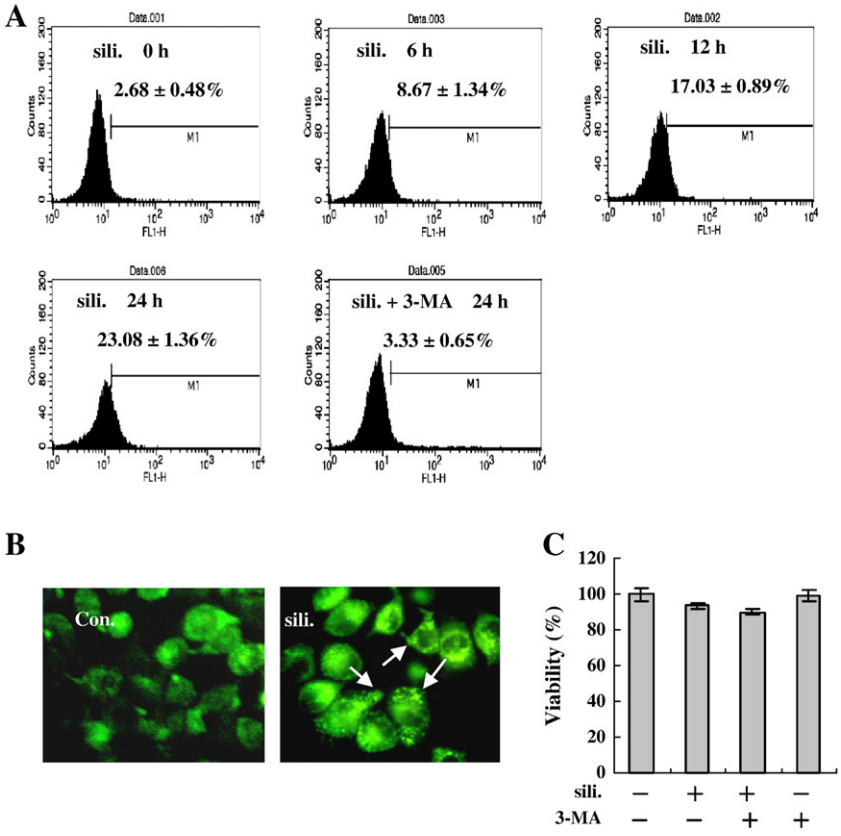

Fig. 2. Silibinin-induced autophagy in A375-S2 cells. The cells were treated with 150 μM silibinin for 0, 6, 12 and 24 h in the presence or absence of 1 mM 3-MA. A) The autophagic ratio in the cells of each group was measured by flow cytomeric analysis of MDC staining as described in Materials and methods. B) The cell viability was determined by MTT assay. C) The formation of autophagic vacuoles in the cells treated with silibinin for 24 h was observed by fluorescent microscopy of MDC staining at the emission wavelength of 525 nm. Arrows indicated the cells that containing autophagic vacuoles.

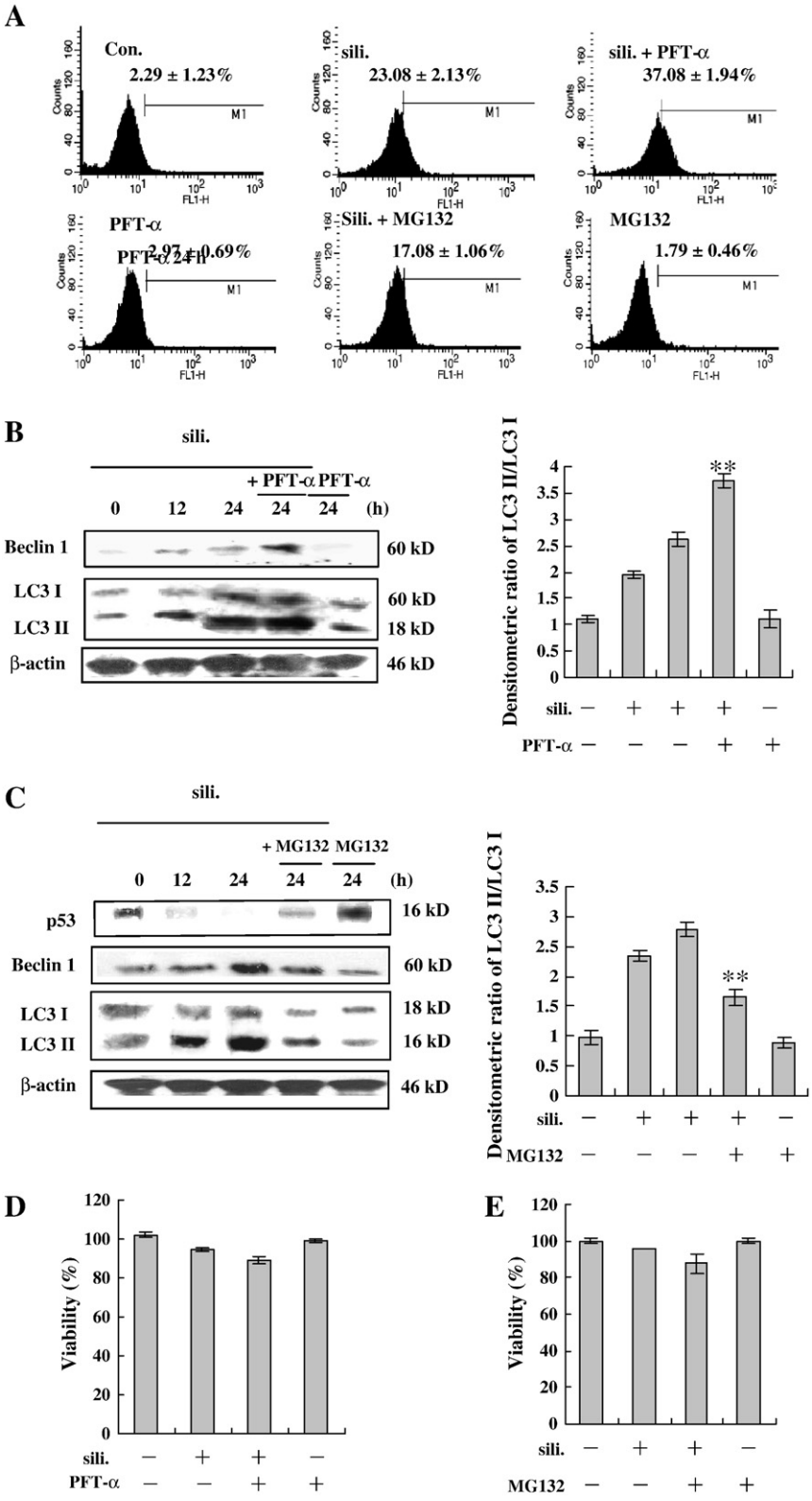

Fig. 3. The pro-autophagic effect of silibinin depended on p53 suppression. (A, D, E). The cells were treated with 150 μM silibinin in the presence or absence of PFT-α (12 μM) or MG132 (0.5 μM) prior to silibinin treatment for 24 h. A) The autophagic ratio in the cells of each group was examined by flow cytometric analysis with MDC. And the cell viability was analyzed by MTT assay (D, E). B) The cells were treated with 150 μM silibinin in the presence or absence of PFT-α (12 μM) prior to silibinin treatment for 24 h. The expression of Beclin 1 was and the conversion of LC3 I to LC3 II (B, left panel) were determined by Western blot analysis, and the densitometric ratio of LC3 II / LC3 I in each group was presented in histogram (B, right panel). **, $p<0.01$. C) The cells were treated with 150 μM silibinin in the presence or absence of MG132 (0.5 μM) prior to silibinin treatment for 24 h. The expression of Beclin 1 and the conversion of LC3 I to LC3 II (C, left panel) were determined by Western blot analysis, and the densitometric ratio of LC3 II to LC3 I in each group was presented in histogram (C, right panel). **, $p<0.01$.

treatment (Fig. 3C, left panel), and accordingly the protein densitometric ratio of LC 3 II / LC 3 I was attenuated by MG132 pre-treatment (1.65 with silibinin and MG132 treated group vs. 2.78 with silibinin treated alone group, $p=0.0014$) (Fig. 3C, right panel). Nevertheless, as shown in Fig. 3D and E, no obvious changes were observed in cell viability in the presence of PFT-α or MG132 ($94.67\pm3\%$ with silibinin treated group vs. $90.55\pm2.12\%$ with silibinin and PFT-α co-treated group, $p=0.1217$; $95.76\pm0.20\%$ with silibinin treated group vs. $91.83\pm4.96\%$ with silibinin and MG132 co-treated group, $p=0.062$), suggesting that in this context, the cytotoxicity of PFT-α and MG132 on cell viability was marginal.

3.4. Silibinin activated NF-κB in p53-dependent manner, and inhibition of NF-κB by PDTC ameliorated silibinin-induced autophagy

In this study, we also found that silibinin up-regulated the protein levels of nuclear factor κB (NF-κB), p-NF-κB (an active form of NF-κB) and p-I-kappaBα (p-I-κBα), and down-regulated the protein level of I-κBα (Fig. 4A). I-κBα being as an inhibitory protein of NF-κB, it blocked NF-κB activation by forming a heterodimer with NF-κB. The phosphorylation of I-κBα releases an active NF-κB (Liang et al., 2004). P53 inhibitor PFT-α, proteasome inhibitor MG132 and NF-κB specific inhibitor PDTC were respectively used to co-treat the cells with silibinin for 24 h, and the expression of NF-κB, p-NF-κB and p53 were assessed by Western blot analysis. The expression of NF-κB and p-NF-κB were increased conspicuously by PFT-α administration (Fig. 4B, a) but were decreased by MG132 administration in silibinin treated cells (Fig. 4B, b). Therefore, we confirmed that silibinin augmented the expression and activation of NF-κB through inhibiting p53 protein levels. However, suppression of NF-κB by using PDTC failed in altering p53 levels (Fig. 4C). To elucidate whether NF-κB plays a role in regulating autophagy, PDTC was used to suppress NF-κB expression, and as shown in Fig. 4C, the autophagic ratio decreased markedly in cells co-treated with silibinin and PDTC ($29.24\pm1.45\%$ with silibinin vs. $10.24\pm1.98\%$ with silibinin and PTDC, $p=0.0051$) (Fig. 4E). Moreover, we induced the over-expression of NF-κB by using LPS (Fig. 4D), and assessed the autophagic ratio by flow cytometric analysis. It turned out that administration of LPS induced a high level of autophagy, and the increased autophagic ratio was decreased by

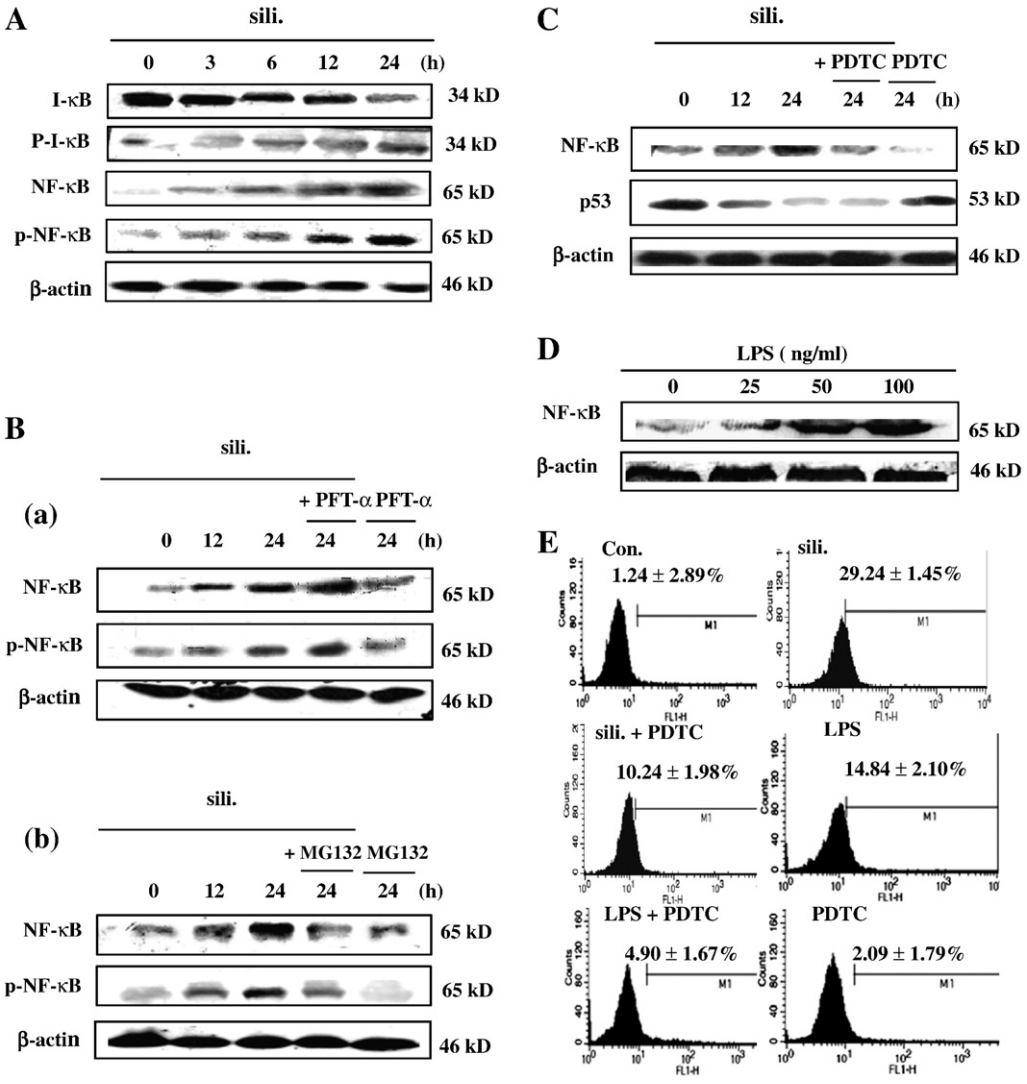

Fig. 4. NF-κB activation-induced autophagy relied on p53 suppression. A) The cells were treated with silibinin at 150 μM for 0, 3, 6, 12 and 24 h before lysis. And the expression of I-κBα, p-I-κBα, NF-κB and p-NF-κB were detected by Western blot analysis. B) The cells were treated with 12 μM PFT-α (a) or 0.5 μM MG132 (b) for 1 h prior to co-incubation with silibinin for 12 and 24 h, then the cells were lysed, and the protein levels of NF-κB and p-NF-κB were examined by Western blot analysis. C) Treatment of the cells with 10 μM PDTC for 1 h prior to silibinin treatment for 24 h then lysed and detection of the protein levels of NF-κB and p53 by Western blot analysis. D) The cells were treated with LPS at indicated concentrations and the protein levels of NF-κB were evaluated by Western blot analysis. E) The cells were pre-treated with 10 μM PDTC or control medium for 1 h and incubated with 150 μM silibinin or 100 ng/ml LPS for 24 h. The autophagic ratio was determined by flow cytometric analysis of MDC staining.

PDTC administration (Fig. 4E) (14.84 ± 2.10% with LPS treated alone group vs. 4.90 ± 1.67% with LPS and PDTC co-treated group, $p = 0.00099$). Thus the up-regulation of NF-κB was required in silibinin and LPS-induced autophagy in A375-S2 cells.

3.5. Silibinin-triggered autophagy antagonized mitomycin C-induced cell apoptosis

Since our previous study already demonstrated that silibinin antagonized DNA damaging reagent mitomycin C-induced p53-dependent intrinsic apoptosis in A375-S2 cells (Jiang et al., 2009), we began to investigate the role of autophagy (promoting survival or facilitating death) in silibinin and mitomycin C co-treated cells. MTT analysis of cell viability revealed that mitomycin C at concentrations ranging from 10 to 60 μg/ml, time-dependently inhibited cell growth (Fig. 5A) and caused the over-expression of p53 (Fig. 5B). Thereafter we pre-treated the cells with 3-MA and then co-incubated the cells with silibinin and mitomycin C for 12 h. The growth inhibition of cells in each group was measured by MTT assay. As shown in Fig. 5C, mitomycin C-induced cell growth inhibition was suppressed by silibinin treatment (55.97 ± 5.82% with mitomycin C treated alone group vs. 17.80 ± 2.60% with silibinin and mitomycin C co-treated group, *, $p = 0.0018$), however, this was reversed by autophagy inhibitor 3-MA pre-treatment (17.80 ± 2.60% with silibinin and mitomycin C co-treated group vs. 32.87 ± 3.76% with silibinin, mitomycin C and 3-MA co-treated group, #, $p = 0.0032$). And the protein levels of p53 and the apoptotic ratio were respectively measured by Western blot analysis and by flow cytometric analysis of PI staining (as described in Materials and methods). As shown in Fig. 5D,

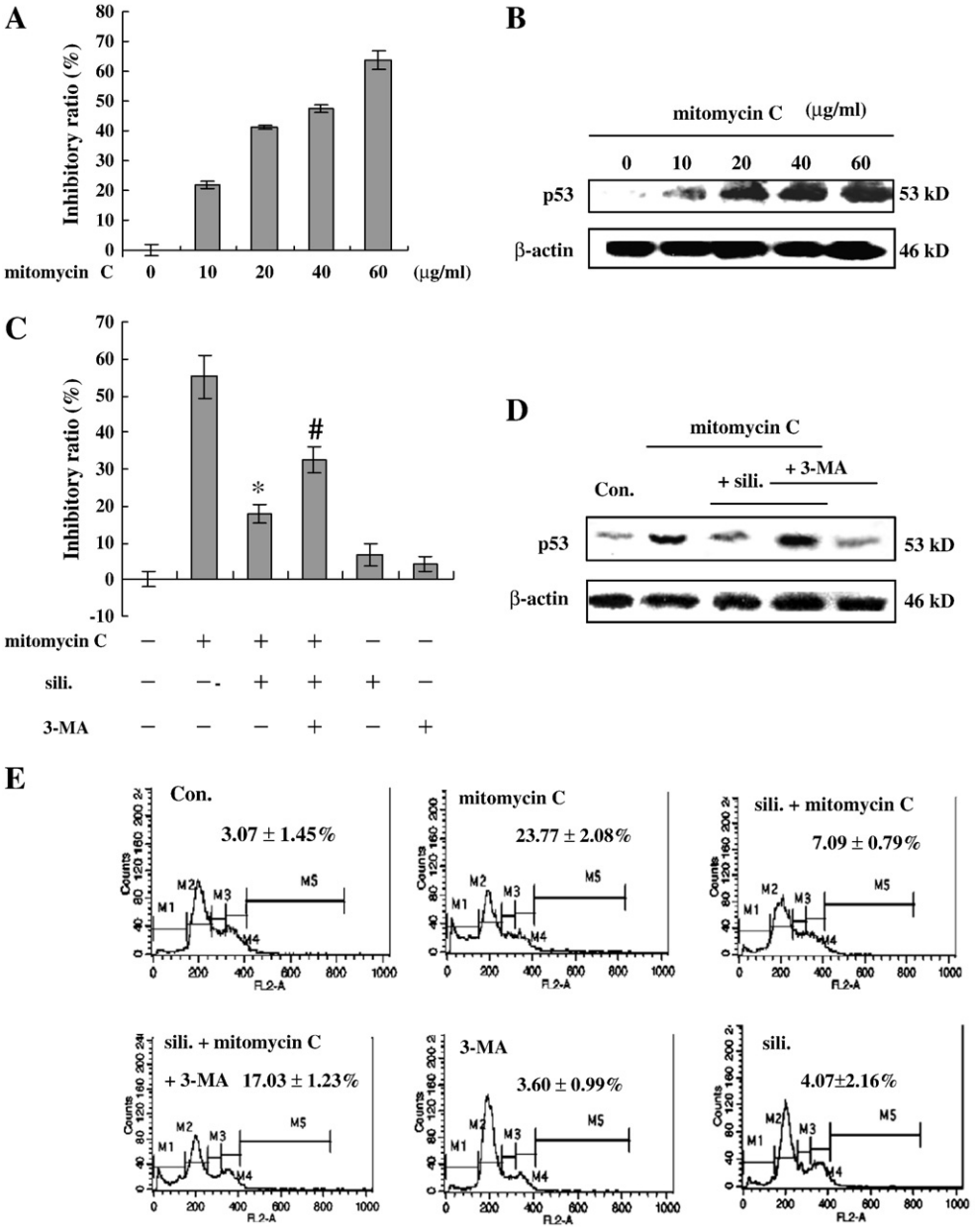

Fig. 5. Suppression of autophagy up-regulated the protein levels of p53. (A, B) The cells were treated with mitomycin C at indicated concentrations for 12 h. The cell viability was examined by MTT assay (A), and the protein levels of p53 were detected by Western blot analysis (B). (C, D) The cells were treated with 1 mM 3-MA for 1 h, and incubated with 150 μM silibinin for 1 h before co-incubation with 40 μg/ml mitomycin C for 12 h. The growth inhibition was measured by MTT assay (C). The cells were collected, lysed, and the protein levels of p53 were detected by Western blot analysis (D).). The cells were collected and fixed, and the apoptotic cells were evaluated by flow cytometric analysis of PI staining. In the analytic diagrams of flow cytometry, the cursors M1, M2, M3, M4, and M5 define the sub-G0/G1 (represents for apoptosis), G0/G1, S, G2/M, and aneuploid phases of the cell cycle, respectively. The percentage of SubG0/G1 was marked in the flow cytometric graph of each group.

3-MA pre-treatment partially abrogated silibinin's suppressive effect on p53 expression. Furthermore, in the cells co-treated with 3-MA, silibinin and mitomycin C, the percentage of cells in sub G0/1 phase (represented as apoptotic ratio) was increased compared to that of silibinin and mitomycin C co-treated cells (17.03 ± 1.23% with 3-MA, mitomycin C and silibinin co-treated group vs. 7.09 ± 0.79% with silibinin and mitomycin C treated group, $p = 0.0027$) (Fig. 5E). Therefore, silibinin-induced autophagy facilitated cell survival in mitomycin C-induced cell insult.

3.6. Blockage of autophagy with 3-MA elevated p53 expression and down-regulated NF-κB expression

The aforementioned results gave a clue that silibinin-induced autophagy by suppressing p53 level, subsequently facilitating the expression of NF-κB. By contrast, our following data showed that the relationship between p53 and autophagy was interactive. Autophagy inhibitor 3-MA pre-treatment led to the escalation of p53 level and the decline of NF-κB and p-NF-κB levels (Fig. 6A). Hence autophagy suppressed p53 expression, thereby augmenting the expression and the activation of NF-κB. Summarizing all the aforementioned results, we drew a conclusion that suppression of p53 by silibinin treatment triggered NF-κB activation and thereby induced autophagy. There is a positive feedback loop between autophagy induction and p53 suppression; namely, p53 suppression-evoked autophagy further accelerated silibinin's suppressive effect on p53 expression. Furthermore, autophagy antagonized mitomycin C-induced cell apoptosis (Fig. 6B).

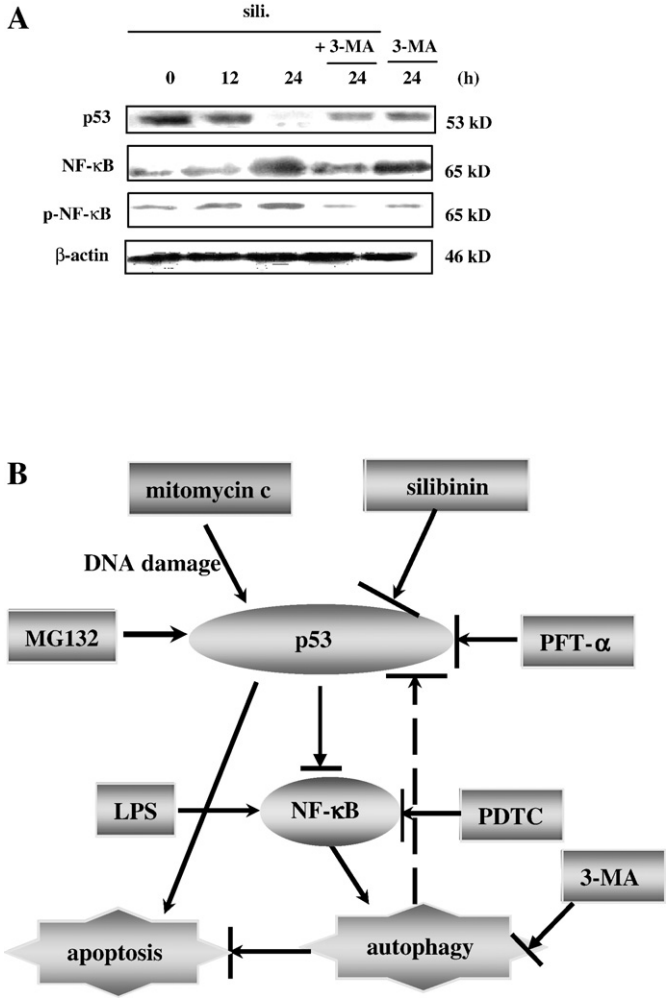

Fig. 6. The regulative effect of autophagy on p53 and NF-κB. A) The cells were treated with 3-MA at 1 mM for 1 h before incubating with silibinin at 150 μM for 24 h. B) The protein levels of p53, NF-κB and p-NF-κB were examined by Western blot analysis. The relationship among p53, NF-κB and autophagy was depicted with a picture.

4. Discussion

Owing to its ideal antiproliferative and anti-apoptotic efficacies in prostate cancer, bladder cancer and breast cancer, silibinin is becoming a hot spot in cancer research. However, our previous studies have reported the anti-apoptotic properties of silibinin in UVB- and mitomycin C-induced A375-S2 cell death models. And these effects are co-related with silibinin's anti-p53 activity (Li et al., 2004; Li et al., 2006). We propose these protective mechanisms are related with its suppressive effect on regulating p53 expression. In accordance with this assumption, the current study has demonstrated that inhibition of p53 evokes the occurrence of autophagy in A375-S2 cells, which is a potential mechanism through which cells avoid being killed by mitomycin C. Besides, in our other study silibinin-induced autophagy is also identified in other cell lines such as in human fibrosarcoma HT1080 cell line and in human epidermoid carcinoma A431 cell line (manuscripts in preparation). And the corresponding autophagy induction mechanisms are still under investigation.

Tumor suppressor p53 plays a major role in safeguarding the integrity of the genome in normal physical condition and in response to a wide range of stress signals. Activation of p53 induces a serial of responses, including cell cycle arrest, apoptosis and senescence. P53 mutations have been documented in more than 50% of cancers and they covered cancers of all tissue origins (Tsao et al., 2004; Meek, 2009). Recently, researchers are focusing on investigating the role of p53 in controlling autophagy since autophagy has been found to be a potent cause of cancer cell resistance to radiotherapy and chemotherapy (Meredith et al., 2010). However, NF-κB as a target of p53 in regulating autophagy has not been reported. In this study we have demonstrated that silibinin induces p53 suppression below cellular basic level and induces autophagy in a time-dependent manner. This finding is in accordance with the work of E. Tasdemir et al., reporting that suppression or knock out of p53 induces autophagy in HCT 116 cells (Tasdemir et al., 2008). PFT-α inhibits the expression of p53, enhances the expression of autophagic associated protein Beclin 1 and facilitates the conversion of LC3 I to LC3 II. Therefore, we suppose that suppressing p53 promotes the occurrence of autophagic process. This is verified by using proteasome inhibitor MG132. MG132 induces p53 accumulation and blunts autophagy, suggesting that the maintenance of basic level of p53 plays a negative role in the control of autophagy. Once p53 levels fall below the cellular basic level, autophagy occurs and this context is reversed by the administration of autophagy inhibitor 3-MA. Therefore, there is a positive feedback loop between p53 suppression and autophagy induction.

The transcription factor NF-κB, more than a decade after its discovery, remains an exciting and active area of study owing to its multiple and conserved functions. These functions include modulating the expression of numerous cytokines and adhesion molecules that were involved in innate or adaptive immunity in the organism's response to infection and stress insults (Liang et al., 2004; Neurath et al., 1998), and manipulating cell survival, death, differentiations and migration (Mayo and Baldwin, 2000). And now there are compelling evidence demonstrating that NF-κB is dysregulated in many forms of cancer and exerts different, even contradictory effects, which depend on different cell types or the variety of stress insults (Baldwin, 2001). In the current study it was found that NF-κB activation is enhanced apparently in the presence of p53 inhibitor PFT-α, and is abolished substantially by inducing p53 accumulation with a proteasome inhibitor MG132 in silibinin treated A375S2 cells. Thus, suppression of p53 precedes and is required for NF-κB activation in silibinin treated A375-S2 cells. PDTC administration fails in altering the suppressive effect of silibinin on p53 expression, demonstrating

that the relationship between p53 and NF-κB is in a one-way-direction. NF-κB has been identified as a negative regulator of autophagy in most conditions (Xiao, 2007; Botti et al., 2006), whereas, the pro-autophagic effect of NF-κB and the correspondent mechanisms are scarcely reported. Our present study has showed that NF-κB inhibitor PDTC efficiently suppresses silibinin-induced autophagy. In addition, LPS, which is able to induce inflammation through activating Toll like receptors, induces NF-κB activation as well as up-regulates autophagy, and this process is also abrogated by PDTC, suggesting that stimulating NF-κB activation either by silibinin or LPS induces autophagy in A375-S2 cells. Results from some other studies also give hints that it may have a positive regulation between autophagy and NF-κB. For example, Delgado et al. have found that autophagy also participates in adaptive immunity responses. Toll like receptors are activated and evoke autophagy in defending extrinsic pathogen. In this context, autophagy accelerates the presentation of antigen peptide to MHC II, which facilitates the maturation of macrophages, promotes the proliferation and differentiation of T cells, and mediates inflammatory responses (Cinel and Opal, 2009; Delgado et al., 2008; Delgado and Deretic, 2009) and all these functions of autophagy are similar to that of NF-κB activation. Hence our findings together with some other results demonstrate that under certain circumstances, NF-κB may function as a mediator of autophagy.

Siwak et al. have found that suppression of NF-κB by curicumin facilitates cell apoptosis in human melanoma cells (Siwak et al., 2005). Therefore, NF-κB activation-mediated autophagy is possible to be a protective mechanism in melanoma cells. And considering our previously study about silibinin's cyto-protective effect against mitomycin C-induced apoptosis in A375-S2 cells (Jiang et al., 2009), we investigate the role of autophagy in regulating cell death and survival by using mitomycin C-induced A375-S2 apoptosis model. It turns out that abrogation of autophagy with 3-MA partially abolishes silibinin's suppressive effects on mitomycin C-induced apoptosis. In another word, autophagy plays a pro-survival role in silibinin antagonizing mitomycin C-induced apoptosis. And this finding is in consistence with the study by Lester M. et al. who have found that induction of autophagy enhances the cyto-protective effect in UVA-activated photosensitizer hypericin treated melanoma cells (Davids et al., 2009).

In summary, in A375-S2 cells it is found that silibinin's suppressive effect on p53 expression facilitates NF-κB activation, and subsequently mediates autophagy, which in turn, plays a pro-survival role in silibinin antagonizing mitomycin C-induced apoptosis. Moreover, there is a positive feedback loop between silibinin-induced autophagy and p53 suppression-dependent NF-κB activation. These findings provide new information about signaling pathway between p53 and NF-κB in regulation of autophagic process, and the suppression of autophagy may shed a light on increasing tumor cell's sensitivity to silibinin in the clinical treatment of cancer.

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