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Inhibiting NF-κB activation and ROS production are involved in the mechanism of silibinin's protection against D-galactose-induced senescence ☆

Qiong Wang [a], Libo Zou [b], Weiwei Liu [a], Wenbo Hao [c], Shin-ichi Tashiro [d], Satoshi Onodera [d], Takashi Ikejima [a,*]

[a] China–Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang, China
[b] School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang, China
[c] School of Animal Husbandry and Veterinary, Shenyang Agricultural University, Shenyang, China
[d] Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo, Japan

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ABSTRACT

Aging is featured by intelligence decline, behavioral disorders and cognitive disability. Autophagy is related to senescent development. In this study, we investigated the roles of NF-κB and autophagy in hippocampal neurons of D-galactose-induced senescent mice, and examined the protective roles of silibinin. Senescence was induced in 6-month-old mice by subcutaneous injection of D-galactose (150 mg/kg/d, for 6 weeks). Silibinin (50 mg/kg/d, intramuscular injection, for 6 weeks) or inhibitors (PDTC, 3-MA or rapamycin, 50 mg/kg/d, subcutaneous injection, for 6 weeks) were given 1 h before D-galactose exposure. Senescent control animals received vehicle for the same time. Ethological analysis, immunofluorescence staining, flow cytometric analysis, western blot and enzyme activity assays were used. Compared with senescent controls, silibinin, PDTC or rapamycin-treated mice showed upregulations of spatial recognition memory ($P<0.05$), cellular oxidoreductase activities ($P<0.05$) and autophagy ($P<0.05$) as well as downregulations of MDA ($P<0.05$) and ROS ($P<0.05$) levels. We propose in D-galactose-induced murine senescence, autophagy is inhibited by NF-κB, inducing the deactivations of celluar oxidoreductases and upregulation of ROS level. The protection by autophagy and the promotion of cellular oxidoreductase activities via inhibiting NF-κB activation and ROS production are involved in the mechanism of silibinin's protection against D-galactose-induced senescence.

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1. Introduction

Brain senescence is characterized by the decline of cognitive function, mainly presenting as deteriorations of visual spatial intelligence, cognitive processing speed, delayed recall, learning capability, attention and immediate memory. According to ethological analysis, senescent animals usually suffer from the impairment of spatial recognition memory and disability of escaping reaction acquisition, showing the deterioration on the cognitive function (Wild et al., 2008).

The senescence mechanism is quite complicated, which results from both internal and external factors. The relative signaling pathways involve p53–p21 pathway (Campo-Trapero et al., 2008) and p16–retinoblastoma (Rb) pathway (Sharpless, 2004). Telomeres are located on the chromosomal terminus, protecting chromosomes against impairment and fusion. The excessive shortening of telomeres may induce DNA lesions and senescent procedure (Yang, 2008). ROS oxidation is the main cause of senescence, and generally, senescence can be postponed by ROS scavengers (Lopez-Torres and Barja, 2008). Excessive ROS production destroys and deactivates by oxidation the abilities of cellular oxidoreductases including superoxide dismutase (SOD), glutathione-peroxidase (GSH-pX) and catalase (CAT). Moreover ROS may induce the p53–p21 pathway and lead to senescence, as a secondary messenger molecule (Burhans and Heintz, 2009). DNA impairment may induce apoptosis or senescence via p53 activation (Ben-Porath and Weinberg, 2005) or p16 pathway (Mimeault and Batra, 2009). Moreover, nutritional or growth factor deficiency induces proliferation inhibition and leads to senescence (Joseph et al., 2009).

Autophagy, as a catabolic process for the degradations of most of inactivated macromolecules and all of the cellular impaired organelles, plays an essential role in the management of cellular survival. It was reported that cellular autophagic function declined as aging developed,

Abbreviations: CAT, catalase; D-gal, D-galactose; GSH-pX, glutathione-peroxidase; ITI, inter-trial interval; 3-MA, 3-methyladenine; MDA, malondialdehyde; MDC, Monodansylcadaverine; NF-κB, nuclear factor-κB; PDTC, pyrrolidine-carbodithioic acid ammonium salt; Rapa, rapamycin; Rb, retinoblastoma; Sb, silibinin; SOD, superoxide dismutase; TRITC, tetramethyl rhodamine iso-thiocyanate.

☆ The experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

* Corresponding author. China–Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Wenhua Road #103, Shenyang 110016, China. Tel./fax: +86 24 23844463.
E-mail address: ikejimat@vip.sina.com (T. Ikejima).

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inducing the accumulations in the cell of impaired or senescent organelles and inactivated proteins and leading to the cellular senescence (Qin and Gu, 2004), especially in cardiac myocytes, neurons or other terminally differentiated cells. In the senescent cardiac myocytes, most of lysosomal enzymes were delivered into lipofuscin-contained lysosomes but less were kept in the cytoplasm for autophagy, leading to the progressive increases of the cellular autophagic function and senescence (Terman and Brunk, 2005).

NF-κB is a transcriptional modulator expressed generally in most of the cells in the body and is concerned with regulations of inflammation-related, immune response-related and other physiological response-related gene expressions (Cai, 2009). It was found that the nuclear binding activity of NF-κB was up-regulated strongly and consistently in heart, liver, kidney and brain of aging mice and rats (Helenius et al., 1996). Moreover, it was reported that NF-κB was activated excessively via knock-down of Sirt-6, which promoted the expression of senescence-related genes and induced senescence while the longevity in mice was extended to normal and many progeria symtoms disappeared after NF-κB was deactivated (Kawahara et al., 2009).

Silibinin, a major bioactive flavanone in milk thistle seeds (*Silybum marianum*), has been accepted as a potent scavenger of most free radicals, such as hydroxyl and peroxyl radicals and hypochlorite ion existing neutrophils (Mira et al., 1994). Silibinin has been also found to up-regulate the activities of SOD and GSH-pX in human red blood cells (Altorjay et al., 1992), recover the immuno-activity in the lymphocytes in patients with alcoholic cirrhosis (Feher et al., 1988), protect blood vessels, and stabilize the membranes via the inhibition to lipid peroxidation. In addition, silibinin has been found to have an effect on free iron-induced tissue damage as an ion chelator (Pietrangelo et al., 1995). Onat in 1999, also found silibinin has evident protection against the aging of the skin through the inhibition of proliferation of skin fibroblasts and may become of great clinical interest, for excess fibroblast proliferation is one of the symptoms of aging (Onat et al., 1999). According to a study from Lu, silibinin prevents amyloid beta peptide-induced memory impairment and oxidative stress in mice (Lu et al., 2009).

In the present study, we investigated the roles of NF-κB and autophagy on D-galactose-induced senescence in mice from the points of the decline of cognitive function, the production of ROS, and the expressions and activations of the senescence-related proteins. We also investigated the primary protective mechanism of silibinin against D-galactose-induced senescence.

2. Materials and methods

2.1. Animals and reagents

Kunming mice weighing 18–22 g were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Animals were given free access to water and normal diet. The experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Silibinin was obtained from Panjin Green Biological (Panjin, China) and dissolved in PEG400. D-galactose was obtained from Sinopharm Chemical Reagent (Shanghai, China). Monodansylcadaverine (MDC), tetramethyl rhodamine iso-thiocyanate (TRITC), 3-methyladenine (3-MA) and NF-κB inhibitor PDTC were purchased from Sigma Chemical (St. Louis, MO, USA). Rapamycin (mTOR inhibitor) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Experimental design

The effects of systemic administration of silibinin or inhibitors were evaluated after Kunming mice were exposed to D-galactose (150 mg/kg/d, subcutaneous injection, for 6 weeks). Silibinin was administrated at 50 mg/kg/d (Lu et al., 2009) and was delivered using intramuscular injection 1 h before D-galactose exposure every day for 6 weeks. Inhibitors were administrated at 50 mg/kg/d and were delivered using subcutaneous injection 1 h before D-galactose exposure for 6 weeks. After 6-week D-galactose exposure, Y-maze test, water-maze test and step-down test were performed. Then mice were sacrificed and the hippocampi were fixed in buffered formalin for fluorescence detection, and immunofluorescence detection or flash frozen in liquid nitrogen and analyzed by immunoblotting, enzyme activity assay and flow cytometry.

2.3. Ethological analysis

2.3.1. Y-maze test

The Y-maze apparatus was made of wood covered with black paper and had three identical arms with an angle of 120° between each of the two arms. Each arm was 30 cm long, 8 cm wide and 15 cm high. The maze was placed in a separate room with a dim illumination and the floor was covered with sawdust to eliminate olfactory stimuli. The protocol consisted of two trials separated by an inter-trial interval (ITI). In the first trial, the mice were allowed to visit only two arms (start arm and other arm) of the maze for 10 min with the third arm (novel arm) blocked. After 1, 2 and 4 h ITI (Wang et al., 2006), the mice had free access to all three arms for 5 min in the second trial (retention) during which the number of visits and the time spent in the three arms were recorded and novelty versus familiarity was analyzed.

2.3.2. Water-maze test

A water-maze test, as described previously (Glynn et al., 2003), was used with some modifications. The apparatus was a 63 cm × 36 cm × 20 cm tank (length × width × height) made of brown Plexiglas and was divided into five parts which were interlaced and connected, forming a zigzag channel. The water contained was to a depth of 10 cm at 22–24 °C. Each mouse was trained to swim through the zigzag maze from initial region to target region 10 times per day with 30 s as an interval and arriving at the target (escape latency) in 20 s as a qualified reflection for 4 days. The escape latency and the qualified reflections per mouse each day were recorded.

2.3.3. Step-down test

The step-down apparatus consisted of a transparent acrylic rectangular chamber (30 cm × 10 cm × 60 cm high) (Hiramatsu et al., 1995), a floor with stainless-cuprum grids 2 mm in diameter at 5 mm intervals and a rubber platform (4 cm diameter and 4 cm height) set on the grid in one corner. Firstly in the acquisition trial the mouse was placed on the platform and habituated surroundings freely for 3 min, and then electric shocks (1 Hz, 500 ms, 36 DC) were delivered to the grid floor for 5 min. When the mouse stepped down from the platform with all four paws on the grid, the electric shock was delivered, which was counted as an error. A retention trial was performed 24 h after the acquisition trial and the time which elapsed until the mouse stepped down from the platform for the first time (the latency), the number of errors made in 5 min and the total shock time for each mouse were recorded.

2.4. Fluorescence staining

Hippocampi were fixed in 10% phosphate-buffered formalin for 10 h at 4 °C, dehydrated in ascending concentrations of ethanol, cleared with xylene, and embedded in paraffin. Then tissue blocks were cut with a rotary microtome, stained with fluorescent compound MDC, incubated in dark at room temperature for 30 min and analyzed with Olympus fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Immunofluorescence staining

Hippocampi were fixed in 10% phosphate-buffered formalin at 4 °C for 10 h, dehydrated in ascending concentrations of ethanol, cleared with xylene, embedded in paraffin and cut with a rotary microtome. The paraffin sections were deparaffinized in zylene, hydrated in descending concentrations of ethanol, blocked with normal serum at room temperature for 30 min, incubated with primary antibody at 4 °C overnight and TRITC-conjugated-secondary antibody at room temperature for 30 min and analyzed with Olympus fluorescence microscope.

2.6. Flow cytometric analysis

Neurons after separated from hippocampus were stained with MDC following the manufacture's protocol, and the ratio of autophagy-positive cells was analyzed immediately by flow cytometry in FACS Analysis Core Facility, Shenyang Pharmaceutical University.

2.7. Western blot

Hippocampi were homogenized in lysis buffer using a Polytron homogenizer and then centrifuged at 14,000×*g*. Supernatants were used to estimate protein concentrations, and 30 to 70 μg protein/sample was applied to a Tris–glycine gel; proteins resolved were transferred on to nitrocellulose membrane and blocked for 1 h with 5% nonfat dry milk. Membranes were incubated with primary antibodies at 4 °C overnight and then with appropriate secondary antibody followed by enhanced chemiluminescence detection (Pierce, Rockford, USA). Western immunoblots were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA) with minimum background.

2.8. Enzyme activity assay

Hippocampi were homogenized in physiological saline and then centrifuged at 14,000×*g*. Supernatants were used to estimate the concentrations of SOD, MDA and GSH-pX according to the manufacturer's instructions with the SOD, MDA and GSH-pX kits (Nanjing, China) and CAT according to the catalase assay method defined by Aebi (Aebi, 1984). Each sample was analyzed in triplicate.

2.9. Statistical analysis

All the presented data were confirmed in at least three independent experiments and expressed as means ± S.D., unless otherwise indicated. One-way ANOVA was used for statistical analysis and $P<0.05$ was considered significant.

3. Results

3.1. Ethological analysis

To investigate the effects of NF-κB and autophagy on the capabilities of spatial recognition and escaping reaction acquisition of D-galactose-induced senescent mice, Y-maze, water-maze and step-down tests were examined.

3.1.1. The Y-maze

As shown in Fig. 1-A, total duration of arm visits in 5 min retention test in the novel arm was significantly increased compared with the start and other arms after 1 h ITI (effect of arm: $F(2, 127)=11.81$, $P<0.001$) and 2 h ITI (effect of arm: $F(2, 127)=10.17$, $P=0.002$). After 1 h ITI, only mice in D-galactose-3-MA-treated group showed no difference in the duration of arm visits between the novel arm and the other arms. Post hoc LSD showed that D-galactose-treated mice spent significantly less time in 5 min retention compared with mice treated with silibinin, PDTC or rapamycin. After 2 h ITI, only mice in D-galactose-treated and D-galactose–3-MA-treated groups showed no difference in the duration of arm visits between the novel arm and the other arms. Post hoc LSD showed that again D-galactose-treated mice spent significantly less time in 5 min retention compared with mice treated with silibinin, PDTC or rapamycin; while D-galactose–silibinin-treated mice spent significantly less time in the retention compared with mice treated with silibinin–PDTC or silibinin–rapamycin.

Percentage of number of arm visits in the novel arm was significantly increased compared with the start and other arms after 1 h ITI (effect of arm: $F(2, 127)=4.93$, $P=0.027$), as shown in Fig. 1-B. After 1 h ITI only mice in D-galactose-treated and D-galactose-3-MA-treated groups showed no difference in the percentage of number of arm visits between the novel arm and the other arms. Post hoc LSD showed that the percentage of number of novel arm visits in D-galactose-treated group were significantly less in 5 min retention than the one in the group treated with silibinin, PDTC or rapamycin. After 2 h ITI, only mice in D-galactose-treated and D-galactose–3-MA-treated groups showed no difference in the percentage of number of arm visits between the novel arm and the other arms. Post hoc LSD showed that the percentage of number of novel arm visits in D-galactose-treated group were significantly less in 5 min retention than the one in the group treated with silibinin, PDTC or rapamycin; while the percentage of number of novel arm visits in D-galactose–silibinin-treated group were significantly less in 5 min retention than the one in the group treated with silibinin–PDTC.

3.1.2. The water-maze

As shown in Fig. 2-A, in the D-galactose-treated group the escape latency was significantly longer and the number of right reflects was less than the one in control group. Compared to the D-galactose-treated group, the escape latency in D-galactose–silibinin-treated, D-galactose–PDTC-treated and D-galactose–rapamycin-treated group were down-regulated and the number of right reflects were up-regulated in 4 days. Compared with D-galactose–silibinin-treated mice, the D-galactose–silibinin–PDTC-treated and the D-galactose–silibinin–rapamycin-treated mice showed shorter escape latency, while the D-galactose–silibinin–3-MA-treated mice showed longer escape latency on days 3 and 4.

3.1.3. The step-down test

As shown in Fig. 2-B, in D-galactose-treated group the latency was significantly shorter, the number of errors was more, and the total time of lightning stroke was longer than the control group. Compared with the D-galactose-treated group, the latency in D-galactose–silibinin-treated, D-galactose–PDTC-treated and D-galactose–rapamycin-treated group was up-regulated, the number of errors was down-regulated, and the total time was down-regulated; while the latency in D-galactose–3-MA-treated group was down-regulated, the number of errors was up-regulated and the total time was up-regulated. Compared with D-galactose–silibinin-treated group, the D-galactose–silibinin–PDTC-treated and the D-galactose–silibinin–rapamycin-treated group showed the longer latency and the shorter total time of lightning stroke; while the D-galactose–silibinin–3-MA-treated group showed the shorter latency, the more number of errors and the longer total time.

3.2. Expressions of p53, p21, Cyclin B1 and Cdc-2

In order to investigate the roles of NF-κB and autophagy in the induction of murine senescence by D-galactose and to examine the effect of silibinin on the murine senescence, the expressions of Cyclin B1 and Cdc-2 were examined by immunofluorescence staining of TRITC and the expressions of p53, phosphor-p53 and p21 were examined by western blot. As shown in Fig. 3, compared with the control group, the degradations of Cyclin B1 and Cdc-2 were

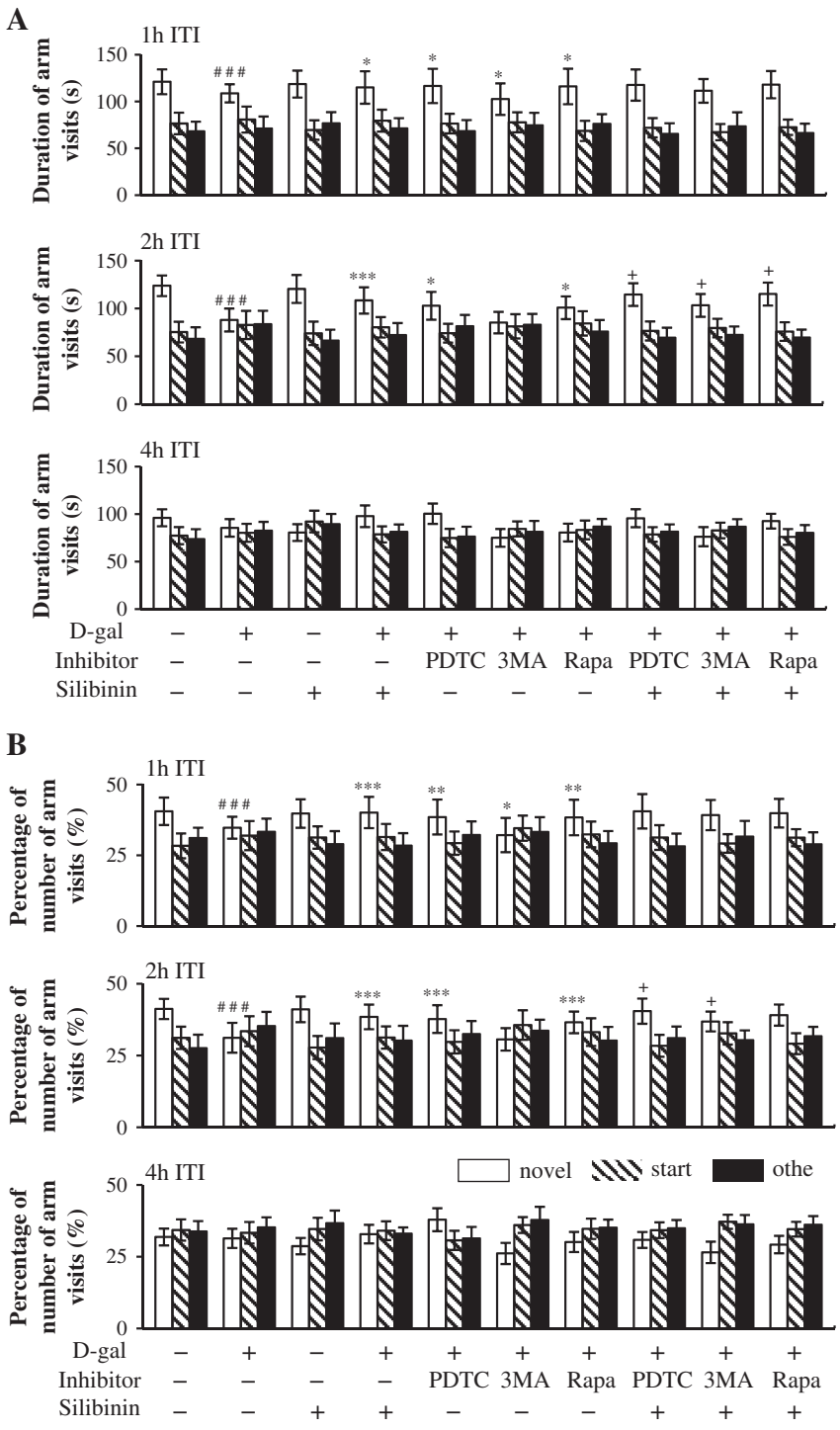


Fig. 1. Roles of NF-κB and autophagy in the D-galactose-induced impaired spatial memory in Y-maze after 1, 2 and 4 h ITI and the effect of silibinin treatment. Panels A and B show respectively mean total duration and percentage of number of arm visits. After 1 and 2 h ITI, D-galactose showed impairment on spatial recognition memory, which was recovered by PDTC, rapamycin and silibinin treatments. No differences between groups were found after 1, 2 or 4 ITI on locomotor activity. Data were expressed as mean ± S.D. $^{###}P<0.001$ for difference in performance in the novel arm between mice treated with D-galactose and controls. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ for differences in performance in the novel arm between mice treated with D-galactose–silibinin, D-galactose–PDTC, D-galactose–3-MA, D-galactose–rapamycin and D-galactose. $^{+}P<0.05$ for difference in performance in the novel arm between mice treated with D-galactose–silibinin–PDTC, D-galactose–silibinin–3-MA, D-galactose–silibinin–rapamycin and D-galactose–silibinin.

suppressed and the expressions of p53, phosphor-p53 and p21 were induced in D-galactose-treated group. Compared with the D-galactose-treated group, the degradations of Cyclin B1 and Cdc-2 were promoted and the expressions of p53, phosphor-p53 and p21 were inhibited in D-galactose–silibinin-treated, D-galactose–PDTC-treated and D-galactose–rapamycin-treated group. The D-galactose–3-MA-treated group showed suppressed degradations of Cyclin B1 and Cdc-2 and the expressions of p53, phosphor-p53 and p21 were induced further. Compared with the D-galactose–silibinin-treated group, the expressions of Cyclin B1 and Cdc-2 and the expressions of p53, phosphor-p53 and p21 were down-regulated in the D-galactose–silibinin–PDTC-treated and the D-galactose–silibinin–rapamycin-treated group; in the D-galactose–

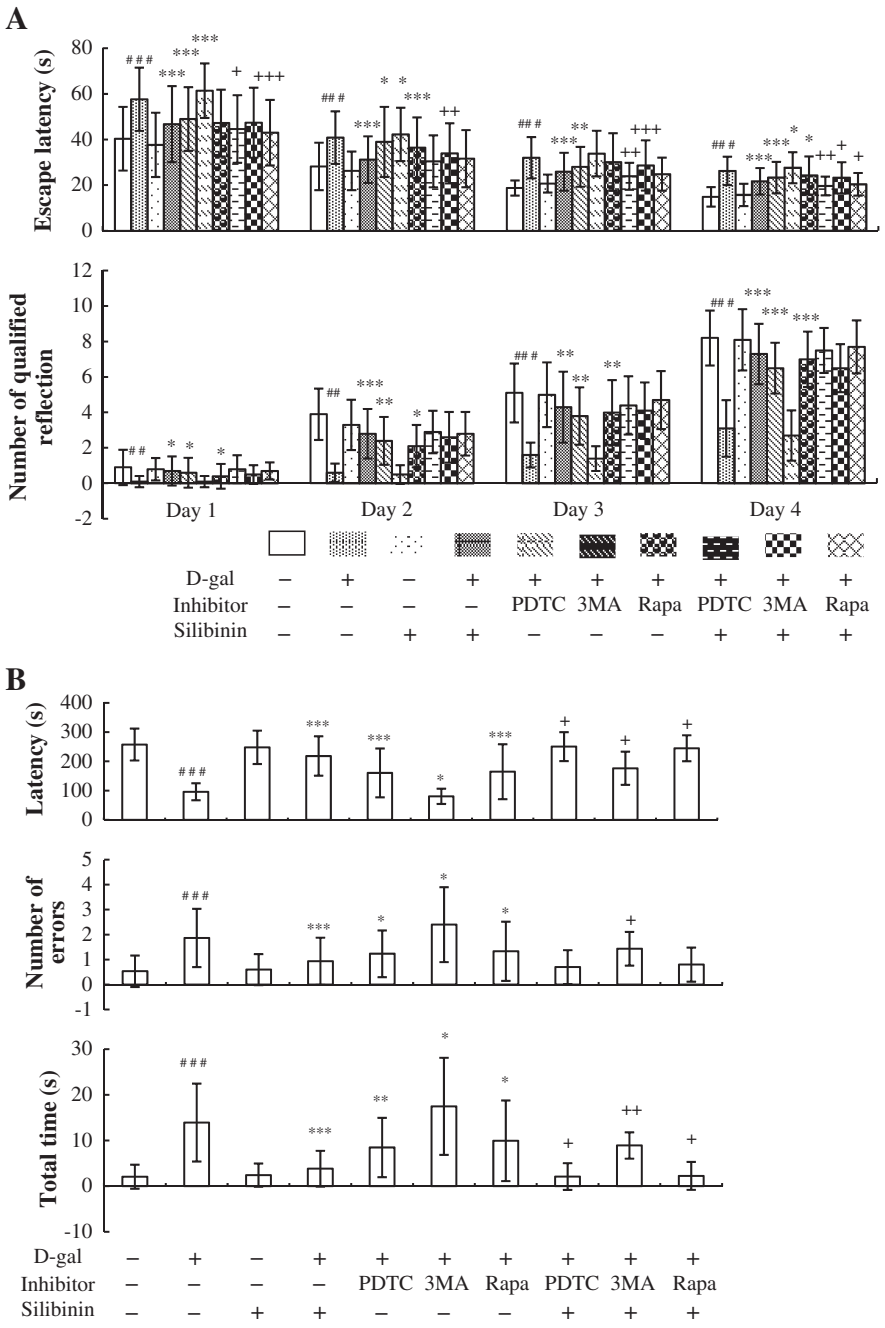
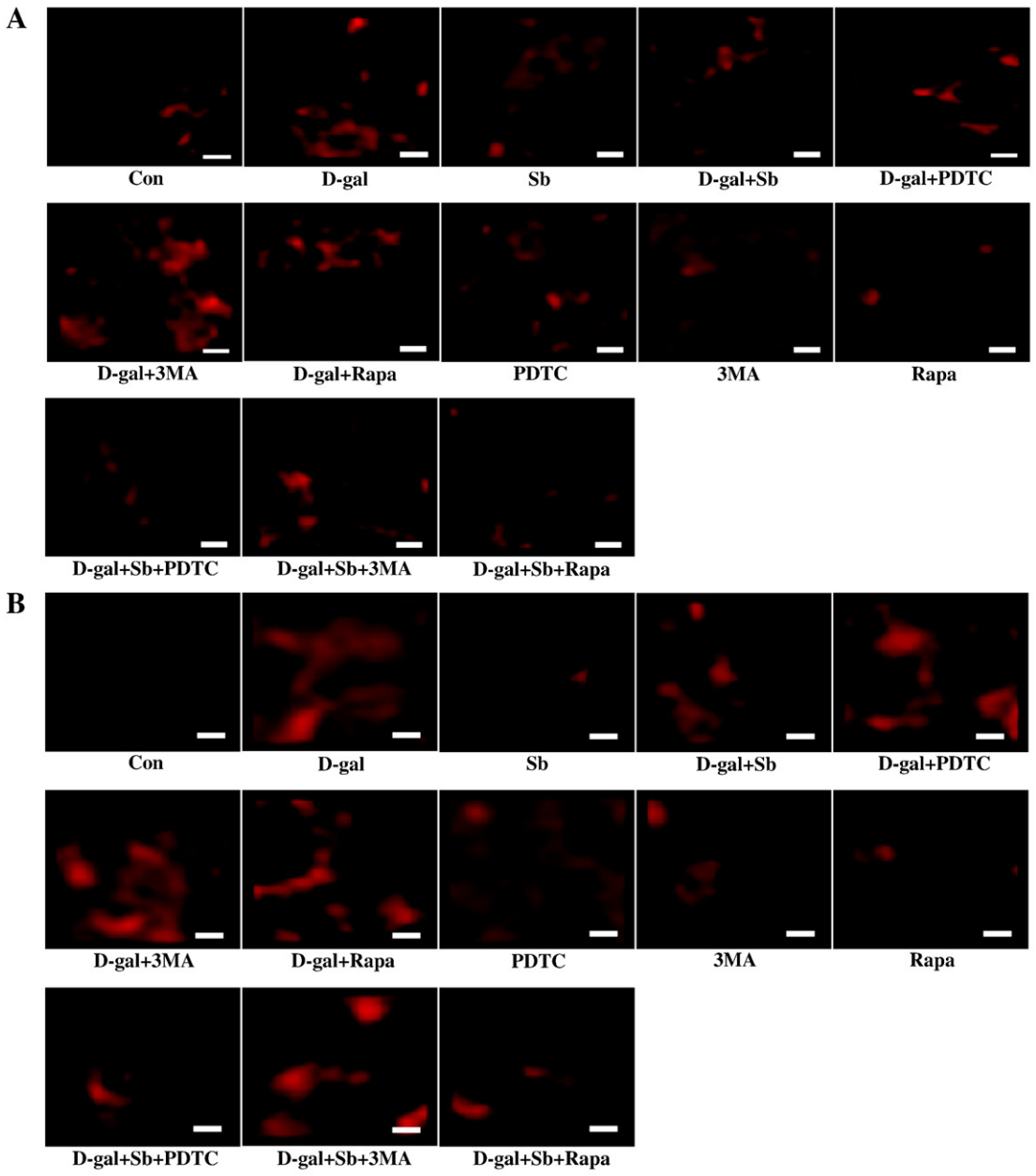


Fig. 2. Roles of NF-κB and autophagy in the D-galactose-induced impaired spatial memory in water-maze and decline of escaping reaction acquisition in step-down test and the effect of silibinin treatment. Panel A shows escape latency and number of right reflects for the murine performance in water-maze while panel B shows latency, number of errors and total time in step-down test. D-galactose exposure to mice induced impairment of spatial memory and decline of escaping reaction acquisition, which was reversed by PDTC, rapamycin and silibinin treatments. Data were expressed as mean ± S.D. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ for differences in performance between mice treated with D-galactose and controls. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ for differences in performance between mice treated with D-galactose–silibinin, D-galactose–PDTC, D-galactose–3-MA, D-galactose–rapamycin and D-galactose. $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ for differences in performance between mice treated with D-galactose–silibinin–PDTC, D-galactose–silibinin–3-MA, D-galactose–silibinin–rapamycin and D-galactose–silibinin.

silibinin–3-MA-treated group, the expressions of Cyclin B1 and Cdc-2 and the expressions of p53, phosphor-p53 and p21 were up-regulated.

3.3. Enzyme activity assays for cellular oxidoreductases

To investigate the effects of NF-κB and silibinin on the redox system in hippocampal neurons of D-galactose induced senescent mice, the activities of SOD, GSH-pX and CAT and the content of MDA were examined as mentioned in Materials and methods; while the ROS content in hippocampus was examined by flow cytometric analysis with DCF-DA. As shown in Fig. 4, compared with the control group, the activities of SOD, GSH-pX and CAT were inhibited and the contents of MDA and ROS were increased in D-galactose-treated group. Compared with the D-galactose-treated group, the activities of SOD, GSH-pX and CAT were promoted and the contents of MDA and ROS were decreased in D-galactose–silibinin-treated, D-galactose–PDTC-treated and D-galactose–rapamycin-treated group. The D-galactose–3-MA-treated group showed suppressed activities of SOD, GSH-pX and CAT, and the contents of MDA and ROS were up-regulated further.



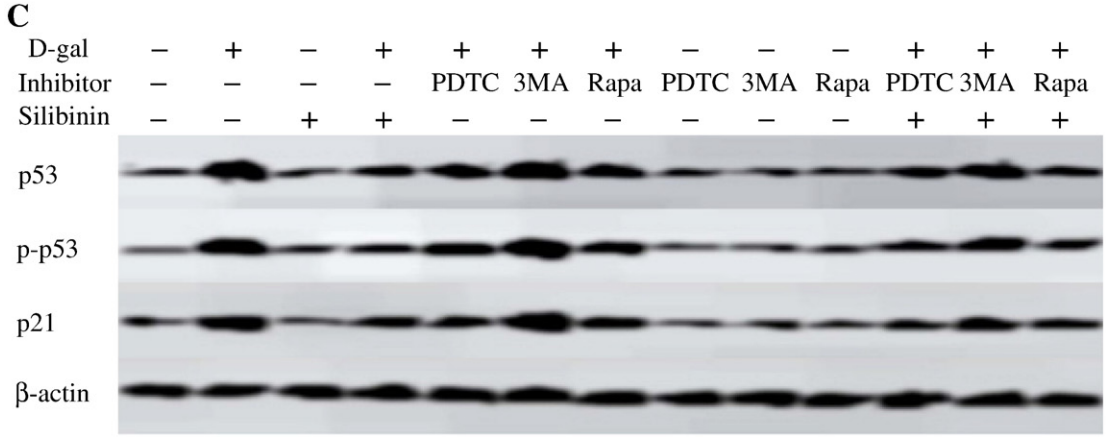


Fig. 3. Effects of NF-κB, autophagy and silibinin on the expressions of senescence-related proteins in the hippocampus of D-galactose treated mice. Panels A and B show the degradations of Cyclin B1 and Cdc-2 in the hippocampal neurons while panel C shows the expressions of p53 and p21. D-galactose treatment suppressed the degradations of Cyclin B1 and Cdc-2 and induced the expressions of p53, phorpho-p53 and p21, which was inhibited by PDTC, rapamycin and silibinin treatments. Membrane was stripped and blotted for β-actin as loading control. Scale bars, 20 μm (A, B).

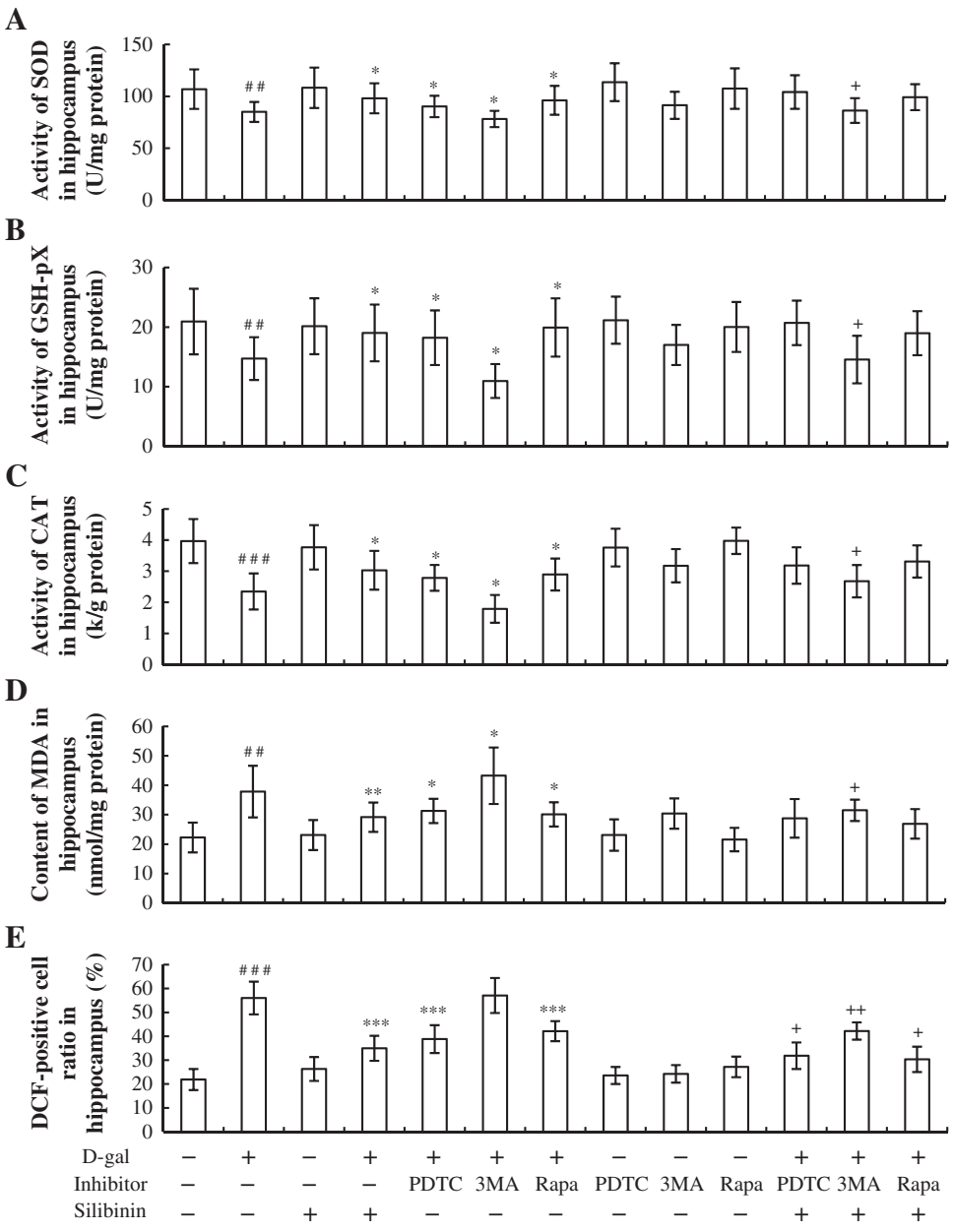


Fig. 4. Effects of NF-κB, autophagy and silibinin on the activities of SOD, GSH-pX and CAT and the contents of MDA and ROS in the hippocampus of D-galactose treated mice. Panels A–C show the activities of SOD, GSH-pX and CAT in the hippocampal neurons while panels D and E show the contents of MDA and ROS. D-galactose treatment inhibited the activities of SOD, GSH-pX and CAT and increased the contents of MDA and ROS, which was reversed by PDTC, rapamycin and silibinin treatments. Data were expressed as mean ± S.D. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ for differences between mice treated with D-galactose and controls. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ for differences between mice treated with D-galactose–silibinin, D-galactose–PDTC, D-galactose–3-MA, D-galactose–rapamycin and D-galactose. $^{+}P<0.05$, $^{++}P<0.01$ for differences between mice treated with D-galactose–silibinin–PDTC, D-galactose–silibinin–3-MA, D-galactose–silibinin–rapamycin and D-galactose–silibinin.

Compared with the D-galactose–silibinin-treated group, the ROS level was down-regulated more in the D-galactose–silibinin–PDTC-treated and the D-galactose–silibinin–rapamycin-treated group; while in the D-galactose–silibinin–3-MA-treated group, the activities of SOD, GSH-pX and CAT were promoted less and the levels of MDA and ROS were decreased less.

3.4. Level of autophagy

To investigate the effects of NF-κB and silibinin on autophagy in hippocampal neurons of senescent mice induced by D-galactose, the autophagic level of hippocampal neurons were examined by fluorescence staining of MDC and flow cytometric analysis with MDC, while the expressions of Beclin-1, LC3-I and LC3-II were examined by western blot. As shown in Fig. 5, compared with the control group, the autophagic ratio was decreased and the expression of Beclin-1 and conversion from LC3-I to LC3-II were inhibited in D-galactose-treated group. Compared with the D-galactose-treated group, the autophagic ratio was increased; Beclin-1 expression and LC3 conversion were promoted in D-galactose–silibinin-treated, D-galactose–PDTC-treated and D-galactose–rapamycin-treated group. Compared with the D-galactose–silibinin-treated group, the autophagic ratio was up-regulated more in the D-galactose–silibinin–PDTC-treated and the D-galactose–silibinin–rapamycin-treated group, while in the D-galactose–silibinin–3-MA-treated group, the autophagic ratio was up-regulated less.

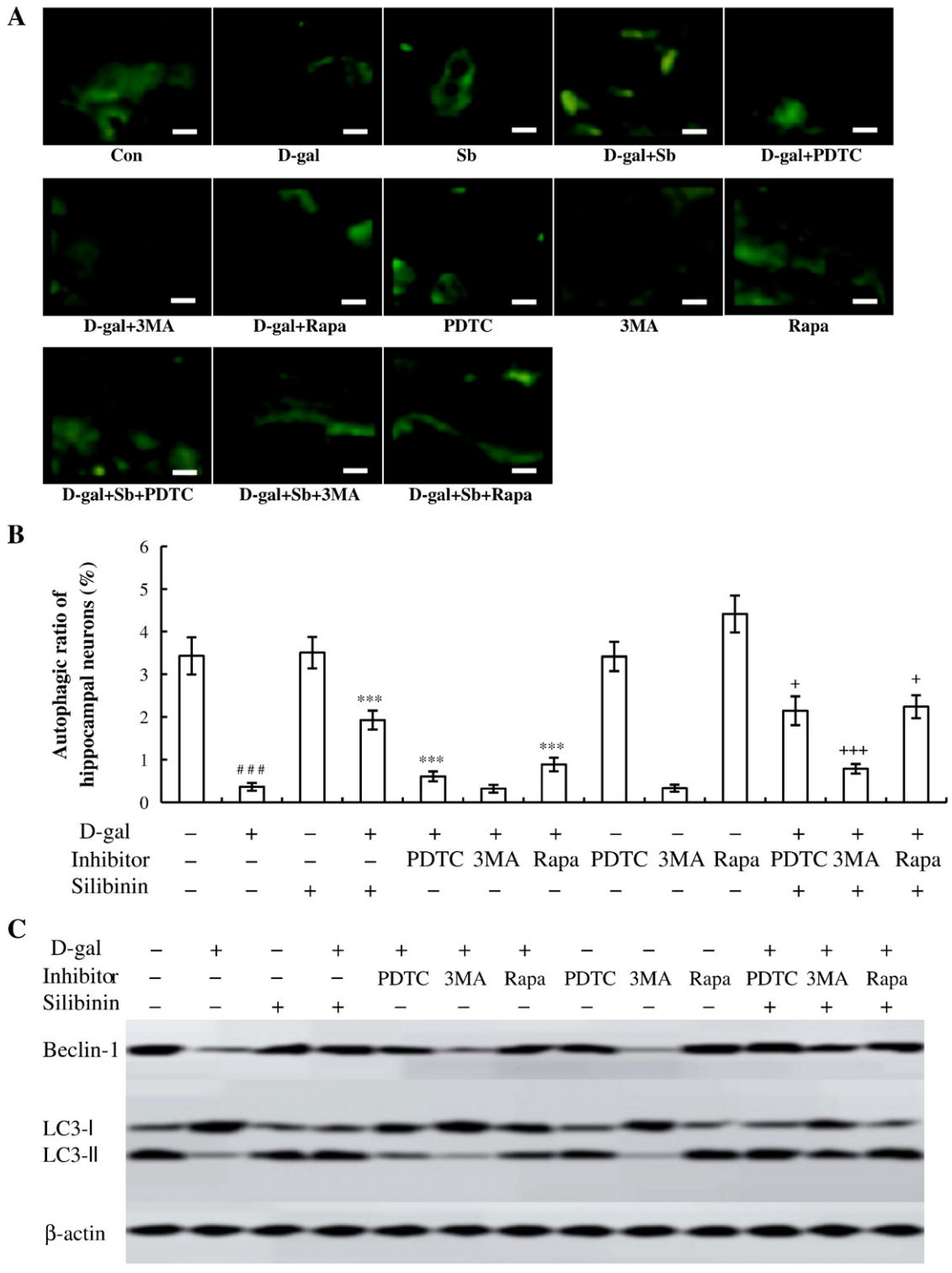


Fig. 5. Effects of NF-κB and silibinin on the autophagic level in the hippocampal neurons of D-galactose treated mice. Panels A and B show the autophagic level in the hippocampal neurons examined by fluorescence staining of MDC and flow cytometric analysis while panel C show the expression of Beclin-1 and the transformation of LC3. D-galactose treatment down-regulated the autophagic level and suppressed the expression of Beclin-1 and the transformation from LC3-I to LC3-II, which was reversed by PDTC and silibinin treatments. Data were expressed as mean ± S.D. $^{###}P<0.001$ for difference between mice treated with D-galactose and controls. $^{***}P<0.001$ for difference between mice treated with D-galactose–silibinin, D-galactose–PDTC, D-galactose–rapamycin and D-galactose. $^{+}P<0.05$, $^{+++}P<0.001$ for differences between mice treated with D-galactose–silibinin–PDTC, D-galactose–silibinin–3-MA, D-galactose–silibinin–rapamycin and D-galactose–silibinin. Membrane was stripped and blotted for β-actin as loading control. Scale bars, 20 μm (A).

3.5. Expressions of NF-κB and I-κB

To investigate the relationship between Sirt-1 and autophagy in D-galactose-induced senescence and the role of NF-κB in the protective mechanism of silibinin, the expressions of NF-κB and I-κB in hippocampus were examined by western blot. As shown in Fig. 6, compared with the control group, the levels of NF-κB and phosphor-NF-κB were increased and the levels of I-κB and phosphor- I-κB were decreased in D-galactose-treated group, compared with which, the levels of NF-κB and phosphor- NF-κB were down-regulated and the

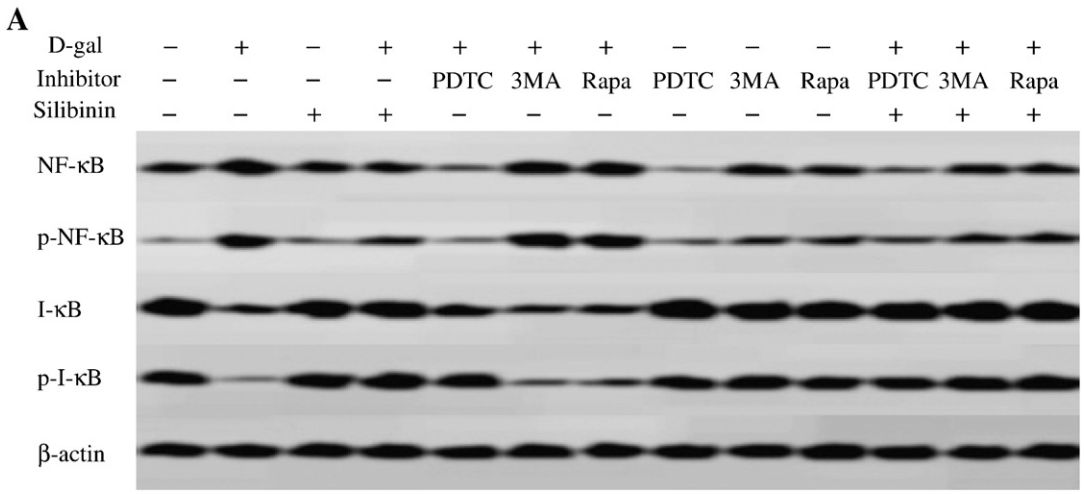


Fig. 6. Effect of silibinin on the expressions and activations of NF-κB in the hippocampus of D-galactose treated mice. Panel A shows the expressions and activations of NF-κB and I-κB in the hippocampus. D-galactose treatment down-regulated the expression and activation of I-κB and up-regulated the expression and activation of NF-κB, which was inhibited by silibinin treatment. Rapamycin or 3-MA showed no effect on the expression and activation of NF-κB. Membrane was stripped and blotted for β-actin as loading control.

levels of I-κB and phosphor- I-κB were up-regulated in D-galactose–silibinin-treated group. Rapamycin or 3-MA showed no effect on the expression and activation of NF-κB.

4. Discussion

In this study, we examined the roles of NF-κB and autophagy in hippocampal neurons in D-galactose-induced murine senescence; assessed the effects of NF-κB and autophagy on murine spatial recognition memory, murine escaping reaction acquisition, the production of ROS and the expressions and activations of the proteins related to senescence in hippocampus of senescent mice; and investigated the protective mechanism of silibinin against D-galactose-induced senescence from the points of NF-κB pathway and autophagy.

The clinical features of senescence are intelligence decline, behavioral disorders and even cognitive disability, including memory deterioration, difficulty in novelty acquisition and cognitive impairment and deficiency. Moreover, the mechanism of the senescence which has been studied for many years, involves telomere shortening (Armanios, 2009), ROS oxidation (Nakanishi and Wu, 2009), DNA impairment (Nakanishi et al., 2009), the activations and expressions of tumor suppressor genes (Fridman and Tainsky, 2008) and others. It was reported that senescence induction and development are associated with NF-κB (Csiszar et al., 2008) and autophagy in neurons (Salminen and Kaarniranta, 2009). Improper adjustment of autophagy may be associated with apoptosis and many diseases such as cancer, neurodegenerative disorders and hepatic encephalopathy (Salminen and Kaarniranta, 2009). Rapamycin induces autophagy by inactivating the protein mammalian target of rapamycin (mTOR) while 3-MA may be regarded as a specific inhibitor of autophagy by suppressing the formation of electron microscopically visible autophagosomes. In this study, PDTC, as the inhibitor of NF-κB, 3-MA and rapamycin were used and the effects of NF-κB and autophagy on D-galactose-induced brain senescence were examined. It was shown that the inhibition of NF-κB and the promotion of autophagy recovered in part murine impaired spatial recognition memory in the Y-maze test and the water-maze test, and escaping reaction acquisition in the step-down test.

There are also significant changes in the molecular biology in senescent cells, as the expressions of pro-senescent and anti-senescent factors are regulated inversely, in which p53, Rb, p21 and p16 play an important role (Ksiazek et al., 2005). So far, we know the signaling pathways to induce senescence include p53–p21 pathway (Bringold and Serrano, 2000) and p16–Rb pathway (Melk et al., 2003). Activated p53 inhibits the degradation of Cyclin B1 and induces Cdc-2 activated constitutively via p21 activation (Hartwell and Kastan, 1994) while the dephosphorylated Rb binds to E2F and suppresses the activity (Pennaneach et al., 2004), which leads to the cell cycle arrest. Protein Rb may be activated via the p16-induced inhibition of Cyclin D/Cdk-4, 6 (Merlo, 2003), or the p21-induced dephosphorylation (Broude et al., 2007), which finally develops senescence. The two pathways could promote one another mutually or only one of them is activated to induce cellular senescence. We examined the expressions of Cyclin B1, Cdc-2, p53 and p21, which demonstrated that the activation of the four senescent markers was associated with the NF-κB activation and the down-regulation of autophagy in the hippocampus of D-galactose induced senescent mice.

ROS, as the primitive cause of many diseases as we know, is related to D-galactose induced senescence (Johnson et al., 1999). DNA and key enzymes proteins could be oxidized by ROS which as the secondary messenger could induce senescence via p53–p21 pathway (Sasaki et al., 2008) and p16–Rb pathway (Wang et al., 2009). In our study, the activities of SOD, GSH-pX and CAT, the three main cellular oxidoreductases, were decreased by ROS oxidation and the content of MDA was increased excessively in the hippocampus of D-galactose induced senescent mice, which was suppressed significantly by the inhibition of NF-κB and the promotion of autophagy. Therefore, D-galactose-induced senescence was caused by excessive ROS production and deactivation of oxidoreductases which, however, was associated with NF-κB activation and down-regulation of autophagy. Meanwhile, silibinin exerted the effect on ROS production via reduction of NF-κB activity and increased autophagy level.

Therefore, we might speculate that murine memory deterioration and cognitive impairment, the activations of the four senescence-related proteins, excessive ROS production and oxidoreductases deactivations were associated with the down-regulation of autophagy and the activation of NF-κB pathway which may promote the apoptosis of hippocampal neurons in D-galactose induced senescent mice. Moreover, it was also shown that the decrease of the cellular autophagic level in the hippocampus of senescent mice was related to NF-κB activation through further investigation. Inhibition of NF-κB activation suppressed D-galactose-induced down-regulation of autophagy while silibinin reversed the down-regulation of autophagy via inhibiting NF-κB activation. The conclusion was backed up by three independent assays including immunefluorescence staining, flow cytometric analysis, and western blot and all the data were clear and coincident to provide evidence in favor the conclusion. NF-κB activation was up-regulated by D-galactose, which mediated the decrease of cellular autophagic level. Silibinin showed the effect on

NF-κB and autophagy regulation in the senescence and helped against senescence.

Silybum marianum (L.) Gaertn (silymarin) is known as a hepatoprotective agent for thousands of years (Mann et al., 2009). The effect of silibinin which is its major constitute, on the D-galactose induced murine senescence was examined in this study. Through ethological analysis, we found that the impaired spatial recognition memory and escaping reaction acquisition of senescent mice were reversed significantly by silibinin administration while the expressions and activations of the four senescence-related proteins, excessive ROS production and oxidoreductases deactivations were inhibited effectively by silibinin in which the antagonism was influenced by NF-κB and autophagy. In addition, the down-regulation of autophagy and the activation of NF-κB in the hippocampus of senescent mice were also suppressed by silibinin administration.

In conclusion, based on the results of the present study, autophagy and NF-κB pathway are involved in the D-galactose induced murine senescence on which silibinin also shows protective efficacy, although more studies are needed in the future to define the pathway of D-galactose induced senescence and the mechanism of silibinin protection.

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