Free Radical Research, July 2011; 45(7): 835–847

informa
healthcare

Role of ROS in the protective effect of silibinin on sodium nitroprusside-induced apoptosis in rat pheochromocytoma PC12 cells

BINBIN LIU[1], PENGFEI YANG[1], YUANCHAO YE[1], YAN ZHOU[1], LINGZHI LI[1], SHIN-ICHI TASHIRO[2], SATOSHI ONODERA[2] & TAKASHI IKEJIMA[1]

[1]*China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang 110016, PR China, and* [2]*Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University; Tokyo 194–8543, Japan*

(Received date: 17 January 2011; Accepted date: 7 April 2011)

Abstract

Silibinin mostly has been used as hepatoprotectants, but it has other interesting activities, e.g. anti-cancer, cardial protective and brain-protective activities. A previous study demonstrated that silibinin protected amyloid β (Aβ)-induced mouse cognitive disorder by behavioural pharmacological observation. This study assessed the effect of silibinin on sodium nitroprusside (SNP)-treated rat pheochromocytoma PC12 cells. Subsequent morphologic observation, flow cytometric analysis and Western blot analysis indicated that treatment with SNP significantly induced apoptosis in PC12 cells. However, silibinin eliminated the apoptotic effect by reactive oxygen species (ROS) generation, especially hydroxyl free radical. Silibinin-induced autophagy through ROS generation when exerting a protective effect and silibinin-induced autophagy also enhanced the ROS generation since 3-methyladenine (3-MA), a specific autophagy inhibitor, decreased the ROS generation and rapamycin, an autophagy inducer, enhanced the ROS generation. Therefore, there exists a positive feedback loop between autophagy and ROS generation. Autophagy prevented SNP-induced apoptosis, since the addition of 3-MA significantly eliminated the protective effect of silibinin. This protective effect was attributed to the generation of ROS and its two downstream Ras/PI3K/NF-κB and Ras/Raf/MEK/ERK pathways. Both prevented PC12 cells from apoptosis. The PI3K/NF-κB pathway induced autophagy to protect PC12 cells, but the Raf/MEK/ERK pathway directly protected PC12 cells bypassing the autophagic effect.

Keywords: *PC12 cells, silibinin, apoptosis, autophagy, ROS*

Introduction

Silibinin, a polyphenolic flavonoid from milk thistle (*Silybummarianum*), has been reported to exert hepatoprotective, cardioprotective and neuroprotective effects [1–4]. However, the mechanism of the neuron protective effect is still not clear. After oral administration of silibinin, its bioavailability is relatively low. However, some studies have found that a complex of phosphatidylcholine and silibinin enhanced bioavailability [5]. The majority of the silibinin presented in plasma was found to be in either the free or conjugate form. Silibinin was eliminated predominantly by glucuronic acid conjugation. The free silibinin possessed the pharmacological activities *in vitro* and *in vivo* [5,6]. Although there were no adequate studies on the brain tissue distribution of silibinin, many studies *in vivo* suggested that the silibinin possessed a neuroprotective effect [7,8].

The rat pheochromocytoma PC12 cells were originally isolated from a tumour in the adrenal medulla of a rat in 1976. Like adrenal chromaffin cells, PC12 cells synthesize and store DA and sometimes noradrenaline, which are released upon depolarization in a Ca^{2+}-dependent way. Therefore, it was widely used as a model for neurons [9]. Some studies reported that undifferentiated PC12 cells could be used as a

Correspondence: Takashi Ikejima, China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Wenhua Road, 110016 Shenyang, Liaoning, PR China. Tel/Fax: +86-24-2384-4463. Email: ikejimat@vip.sina.com

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2011.580343

neuronal cell model aiming to demonstrate the neuroprotective effect of some drugs. Liu et al. [10] reported that a hydrogen peroxide-induced apoptosis model in undifferentiated PC12 cells mimicked oxidative stress-induced cell damage in the brain. Li et al. [11] found that MPP^+-induced undifferentiated PC12 cell apoptosis could mimic the apoptosis in neurodegenerative disorders. Nerve growth factor (NGF) has the potential to elongate neurites or to prevent apoptosis via TrkA, a specific receptor. NGF-differentiated PC12 cells were used as a neurite outgrowth and neurosecretion model [9,12].

Nitric oxide (NO) is a kind of short-lived free radical, which served as a neurontransmitter in many physiological processes such as vasodilation, host defence and synaptic plasticity [13], but high concentration of NO was also involved in many neuronal pathological processes, such as brain ischemia, neurodegeneration and inflammation [14,15]. The NO donor, SNP, has been widely used to study NO-dependent biochemical processes and cell death [16–18]. Therefore, we employed SNP to mimic neuron impairment.

ROS include free radical molecules such as $O_2\bullet^-$, hydroxyl free radical (•OH) and singlet oxygen (•O), as well as non-radical molecules such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [19]. Ferrous Iron (II) is oxidized by hydrogen peroxide to ferric iron (III), a hydroxyl radical and a hydroxyl anion. This reaction was first suggested by Haber and Weiss in the 1930s, but is commonly referred to as 'the Fenton reaction' [20]. In our work, Fenton regent was used as •OH donor. Although •OH has very high reactivity, accumulating data have

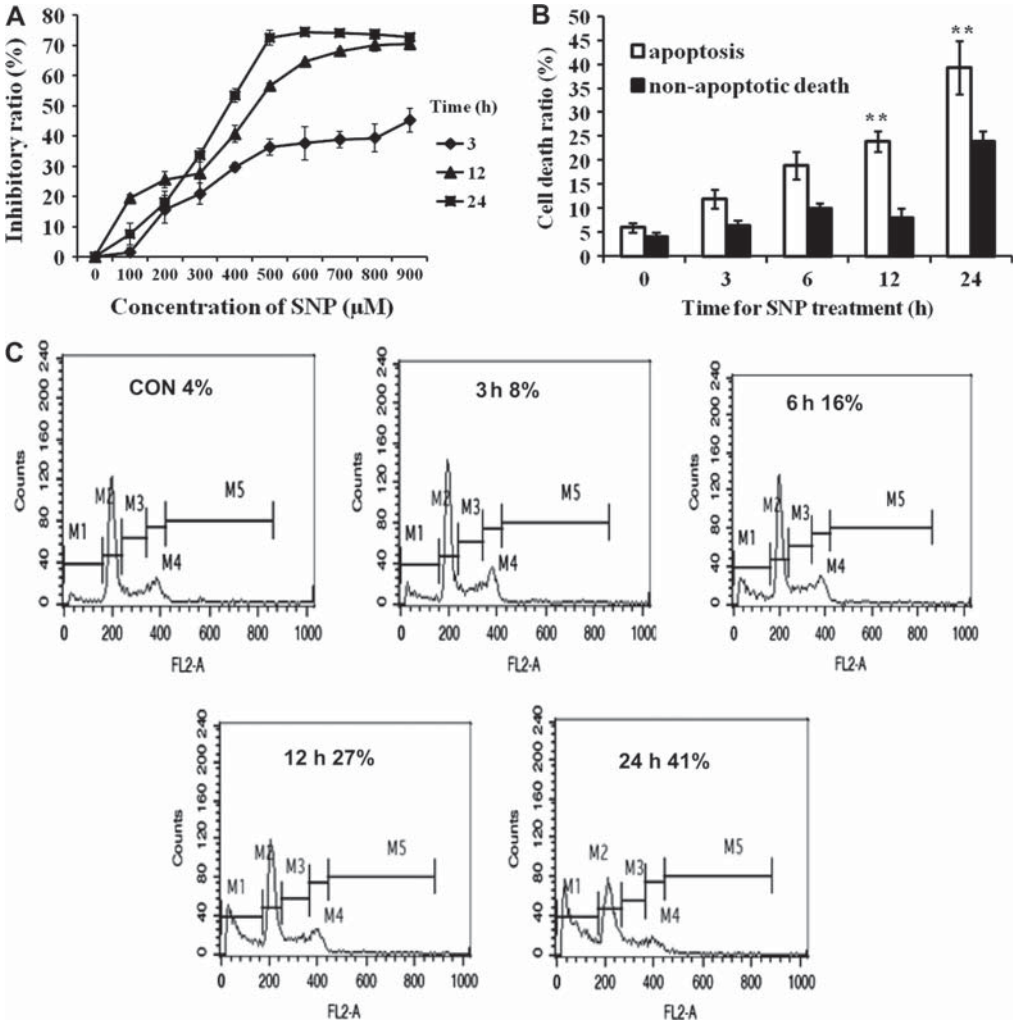
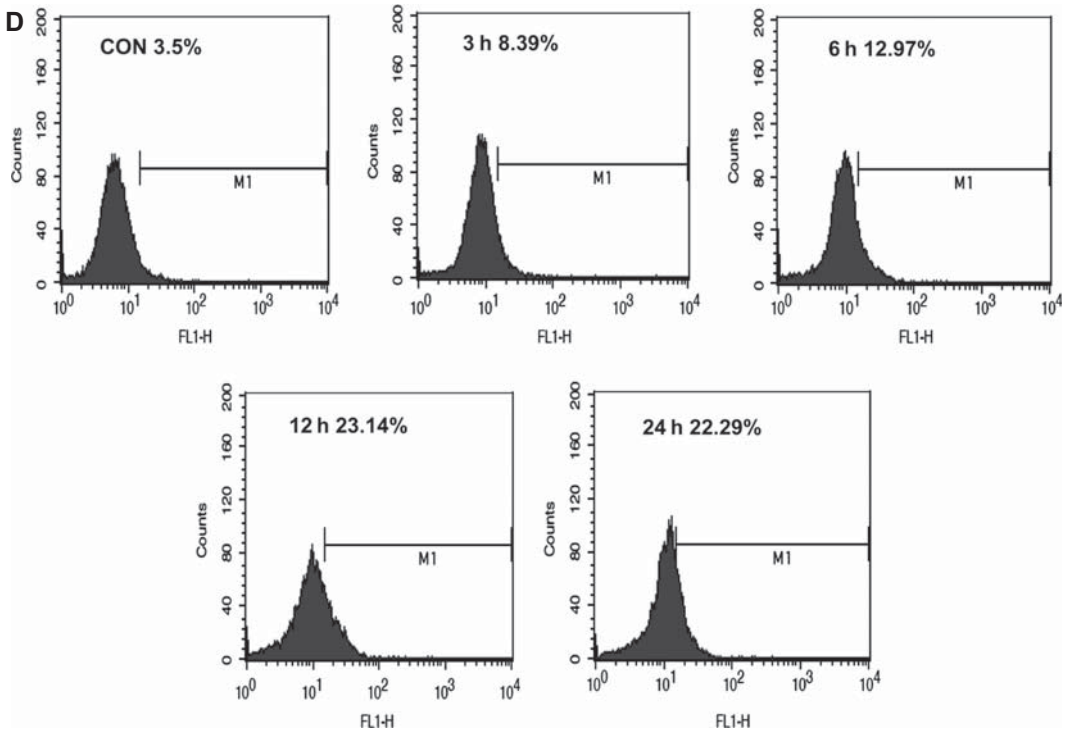
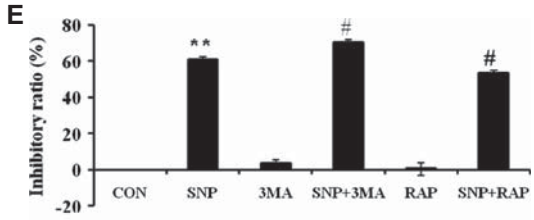


Figure 1. SNP induced PC12 apoptosis in a time- and dose-dependent manner. Inhibitory ratio was measured by MTT assay. PC12 cells were treated with 0–900 μM SNP for 3, 12 and 24 h (A). Characterization of cell death was measured by LDH-based assay. The cells were treated with or without 350 μM SNP for various time periods (B). The apoptosis ratio (C) or autophagy ratio (D) was measured by PI or MDC stained flow cytometry. PC12 cells were treated with or without SNP for 2, 6, 12, 24 h. MTT assay showed the inhibitory ratio when PC12 cells were treated with 350 μM SNP for 12 h and pre-treated with 3 mM 3-MA or 10 μM rapamycin for 1 h (E). Western blot analysis of apoptosis-associated protein express in PC12 cells treated with 350 μM SNP for various time periods (F). $n = 3$, Mean ± SD. $^{**}p < 0.01$ vs control group, $^{\#}p < 0.05$ vs SNP group.





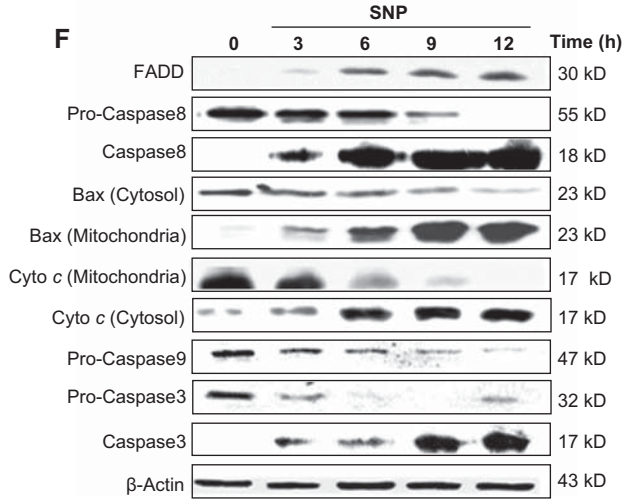


Figure 1. *(Continued)*.

suggested that they also induce *in vitro* cell differentiation and play a more complex role in cell physiology than simply causing oxidative damage [21].

Apoptosis (type I programmed cell death) is a genetically regulated, self-destructive cellular death process that is important in development, tissue remodelling, immune regulation and homeostasis, characterized by cell shrinkage, DNA fragmentation, chromatin condensation and formation of apoptotic bodies [22]. Autophagy, or type II programmed cell death, is also an essential cellular homeostatic mechanism, in which eukaryotic cells degrade wrongly-folded proteins and degenerating cytoplasmic organelles. Under a strict environment, autophagy is rapidly up-regulated to generate energy for survival [23]. The role of autophagy in cell survival during nutrient

depletion and/or in the absence of growth factors has been well established [24]. Now people already found the complex cross-talk between apoptosis and autophagy in deciding cell's fate. Apoptosis often happened along with autophagy. Autophagy has Janus' faces in regulation of apoptosis, which depends on the variety of cell death stimulus [25]. In some circumstances, such as nutrient deprivation or ROS stress, autophagy inhibits apoptosis by engulfing the damaged organelles or misfolded proteins to protect cells [26]. Conversely, stress insult such as menadione and irradiation-induced autophagy facilitates apoptosis. As potential signalling molecules, ROS can regulate apoptosis and autophagy. Therefore, the relationships among them determine cell survival or death. In this work we found that silibinin promoted production of ROS and ROS scavenging repressed the protective effect of silibinin against SNP-induced PC12 cells apoptosis.

Materials and methods

Reagents

Silibinin was obtained from the Beijing Institute of Biologic Products (Beijing, China). The purity of silibinin was measured by HPLC and determined to be ~99%. Silibinin was dissolved in dimethyl sulphoxide (DMSO) to make a stock solution. The concentration of DMSO was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or death. SNP was from Beijing Double-Crane Pharmaceutical Business (Beijing, China). DMEM medium was purchased from GIBCO (Gaithersburg, MD). Foetal bovine serum (FBS) was obtained from TBD Biotechnology Development (Tianjin, China). Propidium iodide (PI), 2′,7′-dichlorodihydrofluorescein diacetate(H2DCFDA),3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), monodansylcadaverine (MDC), acridine orange (AO), Superoxide Dismutase (SOD), N-acetyl-L-cysteine (NAC), catalase (CAT), L-Glutathione reduced (GSH), 3-MA, manumycin A, PD98059, SB 203580, SP 600125, wortmannin and pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma Chemical (St. Louis, MO, USA). Western blot antibodies against FADD, caspase-3, caspase-8, caspase-9, procaspase-3, procaspase-8, procaspase-9, Bax, cytochrome *c*, LC3, Beclin 1, P38, JNK, ERK, p-ERK, Ras, NF-κB, Raf-1, β-actin, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

PC12 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM medium supplemented with 15% foetal 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.

Cell growth inhibition assay

The inhibition of cell growth was measured by MTT assay as previously described [27]. The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well. After 24 h incubation, they were treated with or without silibinin at different concentrations for 12 h. The cells were further co-incubated with SNP for another 12 h at different concentrations. Inhibitors were given 1 h prior to addition of silibinin. Then, 15 μL MTT (5 mg/mL) solution was added to each well and maintained for 3 h. The resulting crystals were dissolved in DMSO. Optical density was measured by MTT assay using a plate microreader (TECAN SPECTRA, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell inhibitory ratio (\%)} = (A_{492\text{control}} - A_{492\text{sample}})/(A_{492\text{control}} - A_{492\text{blank}}) \times 100$$

Preparation of cells

PC12 cells (5×105/well) were seeded into 6-well culture plates. The cells were treated with or without silibinin for 12 h, then further co-incubated with SNP for another 12 h. Inhibitors were given 1 h prior to addition of silibinin.

Observation of morphologic changes

After preparation of PC12 cells, the cellular morphology was observed by phase contrast microscopy (Leica, Nussloch, Germany).

Fluorescence morphologic examination

The PC12 cells prepared were incubated with 10 μg/mL AO or 0.05 mM MDC at 37°C for 30 min. The changes of fluorescence were observed by reverse fluorescence microscopy (Olympus, Tokyo, Japan).

LDH activity-based cytotoxicity assays

Lactate dehydrogenase (LDH) activity was assessed using a standardized kinetic determination kit (Zhongsheng LDH kit; Zhongsheng, Beijing, China). LDH activity was measured in both floating dead cells and viable adherent cells. The floating dead cells were collected from culture medium by centrifugation ($240 \times g$) at 4°C for 5 min and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture supernate (LDHe) was used as an index of non-apoptotic death

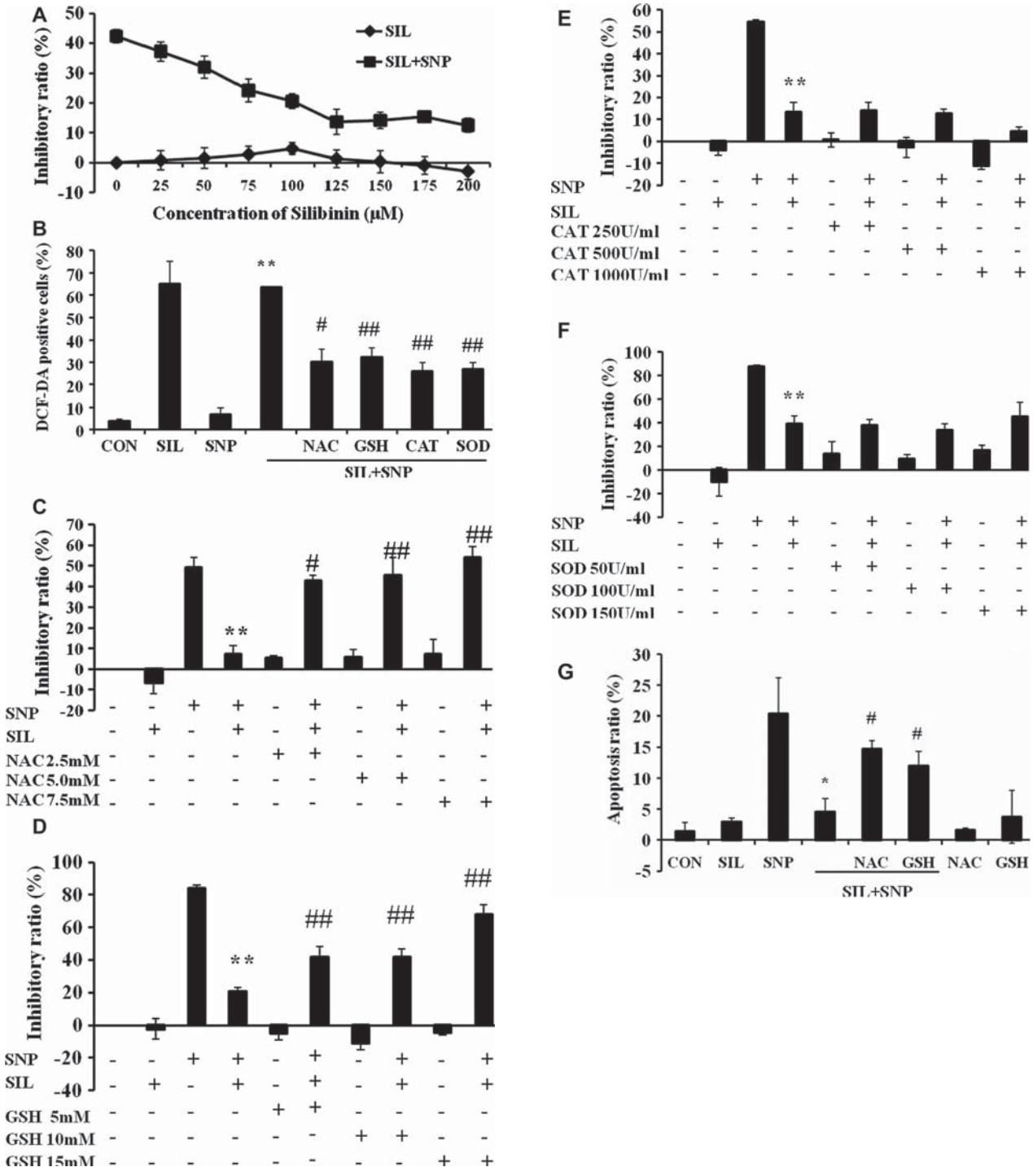


Figure 2. Through ROS, silibinin protected PC12 cells from SNP-induced apoptosis. Inhibitory ratio was measured by MTT assay. The cells were pre-treated with 0–200 μM silibinin for 12 h and then co-incubated with or without 350 μM SNP for another 12 h (A). DCF-DA positive ratio was analysed by flow cytometry (B). Inhibitory ratio of NAC (C), GSH (D), CAT (E) and SOD (F) was measured by MTT assay, respectively. PI flow cytometric analysis (G), morphologic observation (×100 magnification) (H) and AO staining examination (×200 maginfication) (I) indicated that silibinin-induced ROS protected PC12 from SNP-induced apoptosis. The cells were pre-treated with various concentration of •OH free radicals generated by Fenton reaction for 12 h, then further treated with 350 μM SNP. The protective effect of •OH was measured by MTT assay (J). Western blot analysis of apoptosis-associated protein expresses in PC12 cells (K). The cells were treated in the absence of silibinin and SNP (CON), treated with silibinin at 150 μM for 24 h (SIL) and treated with 350 μM SNP for 12 h (SNP) and pre-treated with 150 μM silibinin for 12 h, then co-incubated with 350 μM SNP for another 12 h (SIL + SNP); 7.5 mM NAC (SIL + SNP + NAC), 10 mM GSH (SIL + SNP + GSH), 500 U/mL CAT (SIL + SNP + CAT) and 100 U/mL SOD (SIL + SNP + SOD) were given 1 h prior to addition of silibinin, respectively; $n = 3$, Mean ± SD. $^{**}p < 0.01$ vs control group. $^{*}p < 0.05$ vs control group. $^{\#\#}p < 0.01$ vs silibinin and SNP-co-incubated group. $^{\#}p < 0.05$ vs silibinin and SNP-co-incubated group.

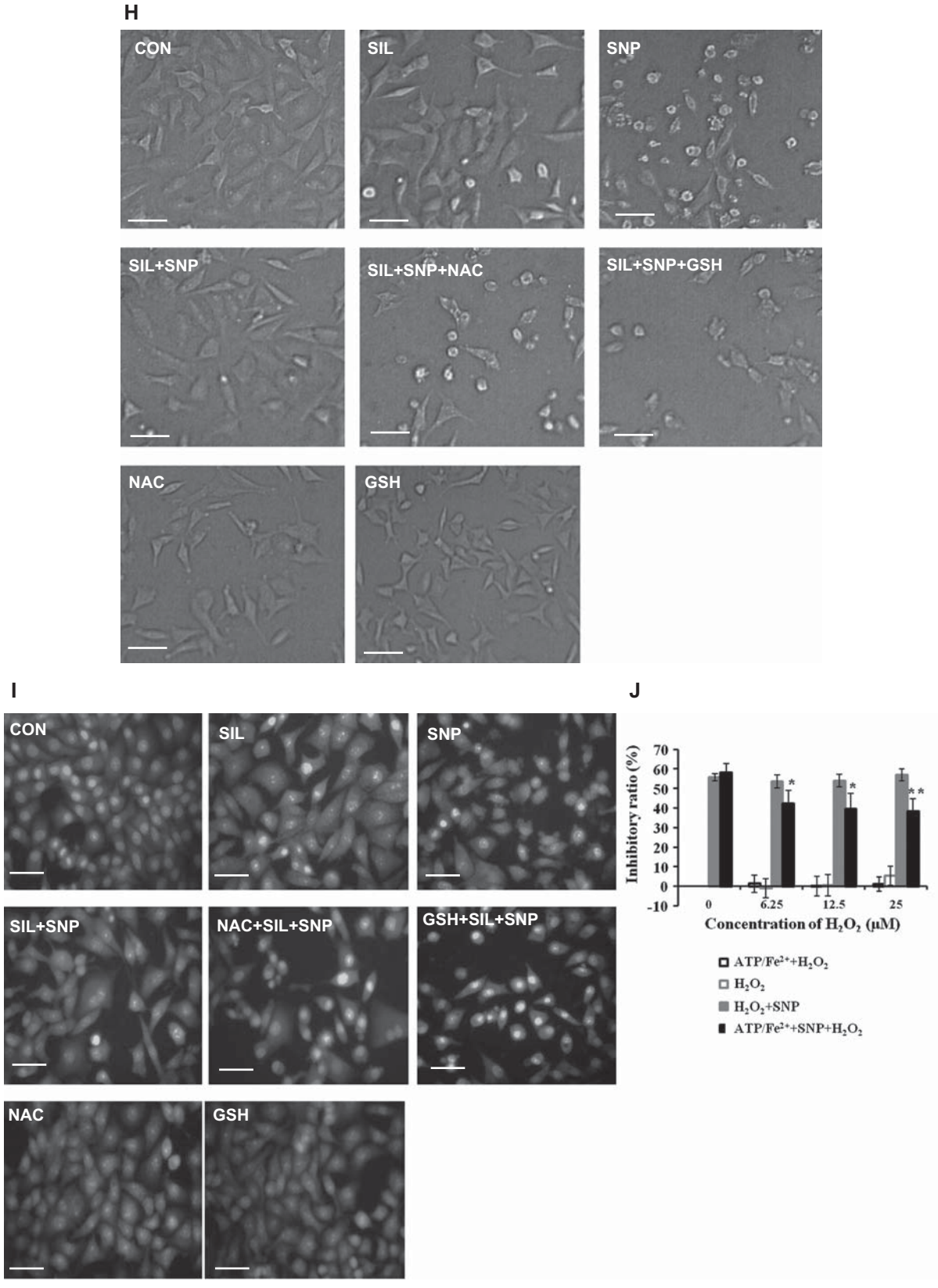


Figure 2. *(Continued)*

and the LDH present in the adherent viable cells was designated as intracellular LDH (LDHi). The percentage of apoptotic and non-apoptotic cell death was calculated as follows:

$$\text{Apoptotic cell death\%} = \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

$$\text{Non-apoptotic cell death\%} = \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

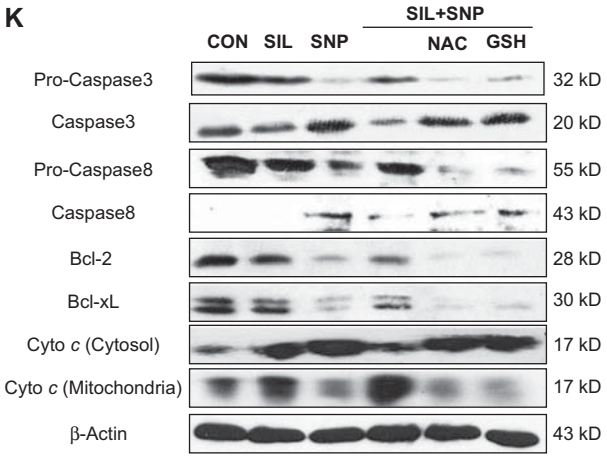


Figure 2. *(Continued)*.

Flow cytometric analysis

For measuring apoptosis, PC12 cells were harvested, rinsed with cold PBS and then fixed in 70% ethanol at 4°C for at least 18 h. Then the cell pellets were stained with the fluorescent probe solution containing 50 μg/mL PI and 1 mg/mL DNase-free RNaseA in PBS on ice in the dark for 1 h. DNA fluorescence of PI-stained cells was evaluated by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

For measuring autophagy, a fluorescent compound, MDC, has been used as a tracer for autophagic vacuoles. PC12 cells were harvested, rinsed with PBS and then stained with 0.05 mM MDC at 37°C for 1 h. After incubation, the cells were washed once with PBS. The samples were analysed by a FACScan flow cytometer.

For measuring ROS generation, thePC12 cells were incubated with 10 mM DCF-DA at 37°C for 30 min. The intracellular ROS mediated oxidation of DCF-DA to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Then the cells were harvested and the pellets were suspended in 1 mL PBS. Samples were analysed by a FACScan flow cytometer.

Western blot analysis

After preparation of PC12 cells, both adherent and floating cells were collected and then Western blot analysis was performed as follows.

For preparation of total proteins, the cell pellets were resuspended in RIPA lysis buffer (Beyotime, Shanghai, China) at 4°C for 60 min. After 13 000 × g centrifugation for 15 min, the total proteins were presented in the supernatant.

For preparation of nuclear and cytoplasmic extraction of proteins, the cell pellets were resupended in lysis buffer A (20 mM HEPES, 10 mMKCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mMPMSF) at 4°C for 60 min. After 28 000 × g centrifugation for 20 min, the cytoplasmic proteins were presented in the supernatant; The remained pellets were resuspended in lysis buffer B (20 mM HEPES, 25% glycerol, 420 mM 1 NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT and 5 μg/mL leupeptin) at 4°C for 15 min. After 16 000 × g centrifugation for 10 min, the supernatant was the nuclear protein extraction [28].

For isolation of mitochondrial and cytosol protein, the cell pellets were resuspended in lysis buffer A (250 nM sucrose, 20 mM HEPES, 10 mMKCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 μg/mL aprotinin and l μg/mL leupeptin) at 4°C for 60 min. After 16 000 × g centrifugation for 30 min, the cytoplasmic proteins were present in the supernatant; The remained pellets were resuspended in lysis buffer B (50 mM, Hepes (pH 7.4), 1% Triton-X 100, 100 mM NaF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) at 4°C for 60 min. After 16 000 × g centrifugation for 30 min, the supernatant was the mitochondrial protein extraction [29,30].

The protein contents of the supernatants were determined by a protein assay reagent (Bio-Rad, Hercules, CA). The protein lysates were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a PVDF membrane. Proteins were detected with indicated primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibody and visualized by a ECL substrate kit (Thermo Scientific, Waltham, MA).

Statistical analysis of the data

All data represent at least three independent experiments and are expressed as the mean ± SD. Statistical comparisons were made by Student's *t*-test. *p*-values of less than 0.05 were considered to represent a statistically significant difference.

Results

Silibinin induced PC12 cell death mainly by apoptosis

SNP caused remarkable inhibition of PC12 cell growth in a time- and dose-dependent manner with an IC_{50} (at 12 h) of 425 μM (Figure 1A). To determine the features of SNP-induced PC12 growth inhibition, LDH assay was carried out. When treated with 350 μM SNP for 12 h, the inhibitory effect was mostly caused by apoptosis, but, at 24 h, the percentage of non-apoptotic cell death was also significantly enhanced (Figure 1B). This result was further confirmed by flow cytometric analysis, indicating that 350 μM SNP induced apoptosis in a time-dependent manner as the percentage of sub-G1 phase increased as time passed (Figure 1C). Then

autophagy was measured by MDC stained flow cytometric analysis, showing that autophagy was also induced by SNP in a time-dependent manner (Figure 1D). MTT assay showed that 3-MA, an inhibitor of autophagy, slightly enhanced the inhibitory ratio of SNP and an autophagy inducer, rapamycin, slightly decreased the inhibitory effect of SNP (Figure 1E). SNP-induced autophagy exerted a minor pro-survival effect on PC12. In this study, we used PC12 cells treated with SNP 350 μM for 12 h as a model of apoptotic cells.

Then, the expressions of apoptosis-associated proteins were examined by Western blot analysis. As shown in Figure 1F, there was a significant and persistent activation of caspase-8 accompanied with a relative increase in the cytostolic adaptor protein FADD after administration of 350 μM SNP, indicating that the Fas death receptor pathway was involved in SNP-induced apoptosis. Furthermore, there was an increased activation of caspase-9, accompanied by the increased release of cytochrome *c* from the mitochondria into the cytosol and the increased transposition of Bax from the cytosol onto the mitochondrial membrane as time passed after the addition of SNP (Figure 1F). The above results indicated that SNP activated both Fas and mitochondrial pathways to induce apoptosis.

Silibinin induced-ROS generation protected PC12 cells from SNP-induced apoptosis

Although silibinin alone showed no significant effects on normal PC12 cells, pre-treatment with this compound showed obvious protective effects at 125 μM or more than 125 μM (Figure 2A). To confirm the generation of ROS, the ROS-specific fluorescent probe H2DCFDA was used. The flow cytometric analysis showed that 150 μM silibinin induced ROS generation and the high amounts of ROS were apparently scavenged by ROS scavenger NAC, $O_2{\bullet}^-$ scavenger SOD, H_2O_2 scavenger CAT or •OH scavenger GSH [31,32] (Figure 2B). This protective effect of silibinin was reversed by NAC and GSH in a dose-dependent manner, but not by CAT and SOD (Figures 2C–F). This result was confirmed by morphologic changes and PI stained flow cytometric analysis (Figures 2G–I). Furthermore, Fenton's reagent, a •OH donor, alone showed no significant effects on normal PC12 cells at low concentration, but pre-treatment with this reagent showed obvious protective effects in a dose-dependent manner (Figure 2J). Western blot analysis showed a similar result. After addition of NAC and GSH, the inhibitory effect of caspase-8, -3, enhancement of Bcl-2, Bcl-xl expressions and a decreased release of cytochrome *c* from the mitochondria into the cytosol induced by silibinin were markedly reversed (Figure 2K).

Through ROS, silibinin induced autophagy, which also enhanced ROS generation in PC12 cells

As autophagy plays an important role in maintaining cellular homeostasis and there is a complex relationship between autophagy and apoptosis, we examined whether autophagy was involved in the protective effect of silibinin on PC12 cells. A significant increase in the number of MDC-labelled fluorescent particles was observed in the silibinin and SNP-coincubated group, but the cells in the control group showed no such particles (Figure 3A). Then MDC stained flow cytometric analysis showed that in the silibinin and SNP-coincubated group, autophagy was induced by silibinin, but NAC and GSH reversed this increase (Figure 3B). Beclin 1, associated with PI3K, has been identified to be responsible for autophagy [33]. Once autophagy occurs, phosphatidylethanolamine is covalently linked to the cytosolic protein LC3-I to yield LC3-II and this conversion is often regarded as a marker for autophagy [34]. Increased expression of beclin1 and the conversion from LC3-I to LC3-II were detected in Western blot analysis (Figure 3C).

Then DCF-DA stained flow cytometry analysis showed 3-MA could decrease the ROS generation and rapamycin enhanced the ROS generation when compared with the silibinin and SNP-coincubated group (Figure 3D). This result demonstrated that there was a positive feedback loop between ROS generation and autophagy.

Silibinin-induced ROS protected PC12 cells from SNP-induced apoptosis by Ras/Raf/MEK/ERK and Ras/PI3K/NF-κB, but silibinin induced autophagy only by Ras/PI3K/NF-κB

MTT assay showed that P38 inhibitor SB 203580 (Figure 4A) or JNK inhibitor SP600125 (Figure 4B) alone had no effect in PC12 cells when compared with the silibinin and SNP-coincubated group. However, Ras inhibitor manumycin A, MEK inhibitor PD 98059, PI3K inhibitor wortmannin, NF-κB inhibitor PDTC and 3-MA reversed the protective effect of silibinin (Figure 4C), indicating that Ras, ERK, PI3K and NF-κB exerted a protective effect.

Western blot analysis indicated that the inhibition of Ras by manumycin A inhibited the enhancement of Raf-1 and ERK and the conversion of NF-κB from cytosol to nuclear was also inhibited. Inhibition of ERK by PD98059 showed no effect on the enhancement of Raf-1 and the transposition of NF-κB except for conversion from ERK to p-ERK. The addition of wortmannin and PDTC, inhibitors of PI3K and NF-κB, respectively, both reversed the transposition of NF-κB from cytosol to nuclear, but showed no effect on the expression of Raf-1 and p-ERK (Figure 4D). The conclusion from the above results was that Raf-1/MEK/ERK and PI3K/Akt are two

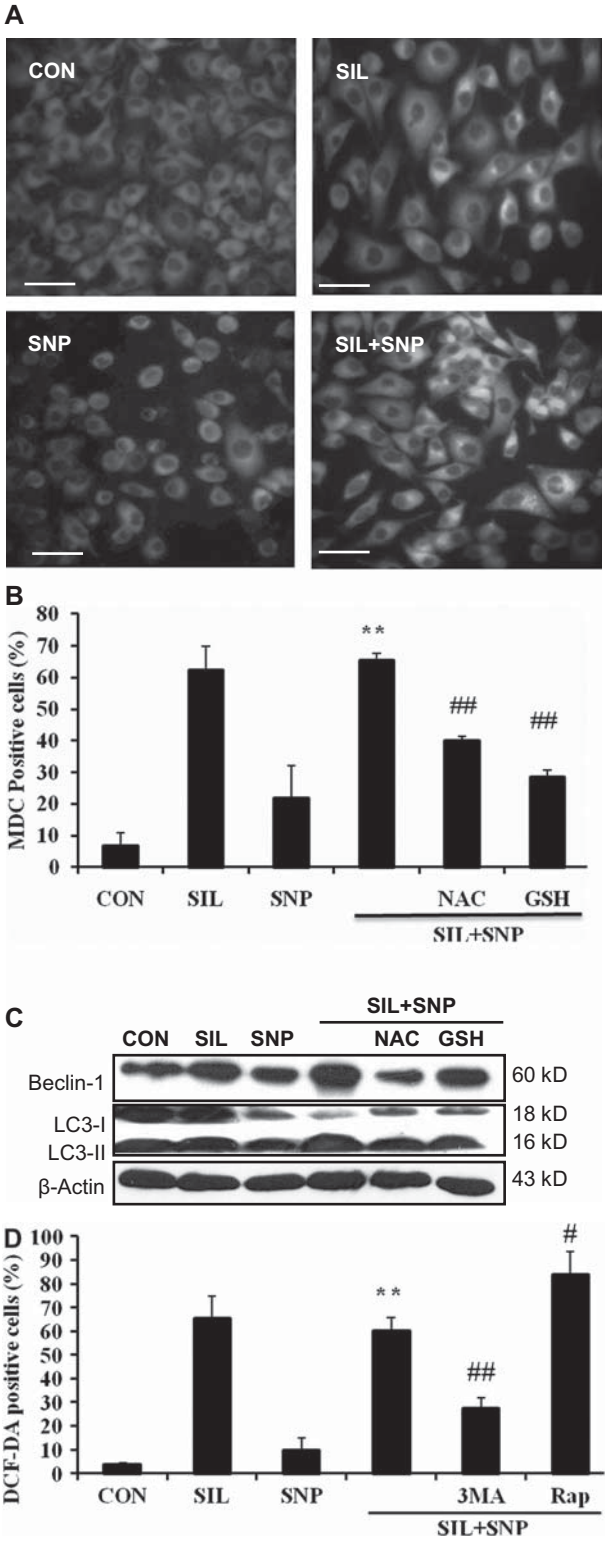


Figure 3. Silibinin induced autophagy by ROS generation and silibinin-induced autophagy enhanced ROS generation in PC12 cells. MDC staining morphologic observation (×200 magnification) (A), MDC stained flow cytometric analysis (B) and Western blot analysis of Beclin 1 and LC3 (C) were performed in PC12 cells. DCF-DA positive ratio was analysed by flow cytometry (D) 3 mM 3-MA (SIL + SNP + 3-MA) and 10 μM rapamycin (SIL + SNP + Rap) were given 1 h prior to addition of silibinin, respectively. The details of other groups are shown in Figure 2; $n = 3$, Mean ± SD. $^{**}p < 0.01$ vs control group. $^{*}p < 0.05$ vs control group. $^{\#\#}p < 0.01$ vs silibinin and SNP-co-incubated group. $\#p < 0.05$ vs. silibinin and SNP-co-incubated group.

independent pathways and Ras was an upstream regulator for these two pathways.

In order to determine the roles of these two pathways in autophagy, the effects of manumycin A, PD 98059, wortmannin, 3-MA and PDTC were examined by MDC stained flow cytometric analysis. The result showed that manumycin A, wortmannin, 3-MA and PDTC reversed the enhancement of autophagy induced by silibinin. However, PD 98059 had no such effect (Figure 4E). Then an inhibitor of autophagy, 3-MA, reversed the protective effect of silibinin, showing that autophagy plays an anti-apoptotic role (Figure 4C). The expression of beclin-1 and the conversion from LC3-I to LC3-II also demonstrated the same result (Figure 4F). The conclusion was that silibinin induced autophagy through the Ras/PI3K/NF-κB but not through the Ras/Raf/MEK/ERK pathway.

Then NAC and GSH decreased the expression of ERK, NF-κB and Ras when compared with the silibinin and SNP-coincubated group (Figure 4G). The ROS might be upstream of both pathways (Figure 4H).

Discussion

In this work, we demonstrated that silibinin-induced ROS generation and autophagy protected PC12 cells from SNP-induced apoptosis. Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment of cognitive function [35]. Common pathological features of AD are senile plaques, neurofibrillary tangles and neuronal loss in the medial temporal lobe structures and cortical areas of the brain [36]. It has been confirmed that peroxynitrite-mediated damage contributes to AD-induced neuronal toxicity and cognitive deficits [37] and is widespread in the brain of AD patients [38]. On the other hand NO can be released from microglia, challenged by Aβ or lipopolysaccharide (LPS). Microglia activation and NO caused great damage to neurons [39]. Therefore, apoptosis induced by SNP was applied to mimic neuron impairment in neurodegenerative disorder, such as AD. Silibinin had been reported to inhibit over-expression of iNOS and increase of nitrotyrosine levels in the brain of AD model mice [40]. Furthermore, silibinin had a protective effect in D-galactose-induced senescent mice [7]. In this work, silibinin reversed SNP-induced apoptosis in PC12 cells. Taken together, these results suggested that silibinin could protect neuron cells from NO-induced neuron damage *in vitro* and *in vivo*.

Many studies found that silibinin exerted an antioxidant effect in the brain [41], human monocytes [42] and others. However, in some researches, silibinin could also induce ROS generation in human fibrosarcoma HT1080 [43] and cancer MCF-7 cells [44]. In this research, we found that silibinin induced

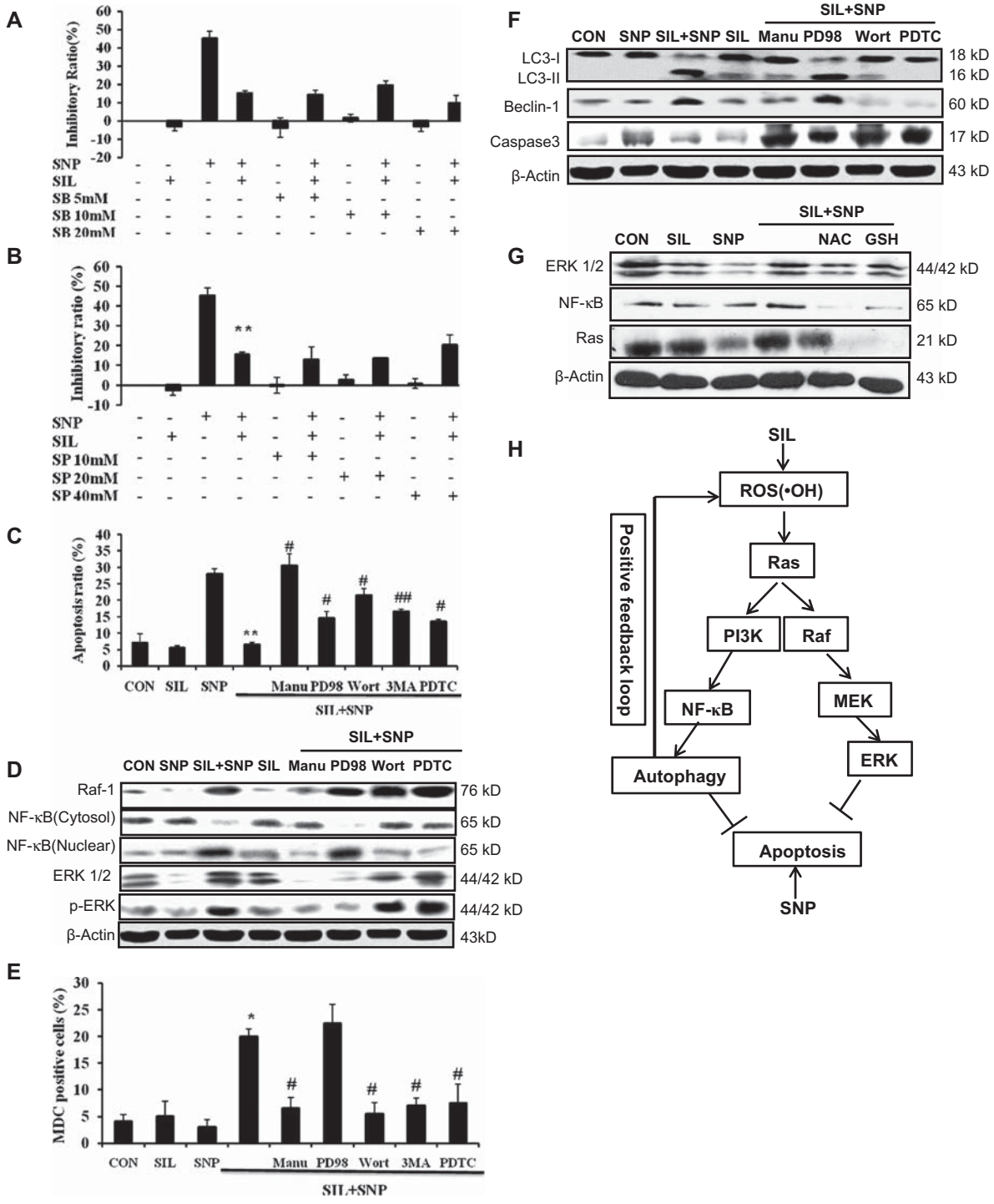


Figure 4. Silibinin-induced ROS protected PC12 cells from SNP-induced apoptosis through Ras/Raf/MEK/ERK and Ras/PI3K/NF-κB pathways, but silibinin induced autophagy only through Ras/PI3K/NF-κB. Inhibitory ratio of SB 203580 (A) and SP 600125 (B) were measured by MTT assay. Apoptosis ratio was measured by PI staining flow cytometry (C). The expression of Raf-1, NF-κB, ERK and p-ERK was measured by Western blotting assay (D). MDC stained flow cytometric analysis (E) and Western blotting analysis of Beclin 1 and LC3 (F) were performed in PC12 cells. Western blotting of ERK, NF-κB and Ras indicated that ROS might be upstream of both pathways (G); 10 μM manumycin A (SIL + SNP + Manu) and 10 μM PD 98059 (SIL + SNP + PD98), 10 μM wortmannin (SIL + SNP + Wort) and 900 nM PDTC (SIL + SNP + PDTC) were given 1 h prior to addition of silibinin, respectively. The details of other groups are shown in Figures 2 and 3. (H) Schematic representation of the protective effect of silibinin-induced ROS in PC12 cells; $n = 3$, Mean ± SD. $^{**}p < 0.01$ vs control group. $^{*}p < 0.05$ vs control group. $^{\#\#}p < 0.01$ vs silibinin and SNP-co-incubated group. $^{\#}p < 0.05$ vs. silibinin and SNP-co-incubated group.

protective ROS generation. The protective ROS provoked by silibinin emerges as •OH, since NAC and •OH scavenger GSH reversed the protective effect of silibinin, but the H_2O_2 scavenger catalase and $O_2^{\bullet -}$ scavenger SOD did not. Furthermore, •OH donor Fenton reagent decreased the inhibitory effect of SNP. Silibinin-induced •OH exerted a protective effect by inhibiting both Fas and mitochondrial

pathways. •OH was reported to induce protein oxidation and neuron damage in rat brain [31,32]. However, the membrane hypothesis of ageing (MHA) assumes that the •OH exert a sort of useful cross-linking in the premature, blast-type cells, which is necessary for the stabilization of the biological structures and the maintenance of a proper functionality of the cell structure. MHA explains cell maturation and ageing on the basis of the intrinsic physicochemical and biochemical interactions between the cell components and basic oxidative processes of the living systems. This hypothesis suggests that •OH free radicals may play a physiologically useful and important role in growth and maturation of the organisms. An experimental testing of this hypothesis on human HL-60 myeloid and K562 erythroid leukaemia cells has already given encouraging results [45,46]. Oravecz et al. [21] also found that hydroxyl free radicals could induce PC12 cell differentiation and that they played a more complex role in cell physiology than simply causing oxidative damage.

Studies in model organisms have implicated autophagy as a crucial regulator of the ageing process. Accumulation of ubiquitylated protein aggregates can occur during normal human brain ageing and reaches pathological levels in neurodegenerative disorder such as AD [47]. Our previous study found that silibinin protected against D-galactose-induced senescence through autophagy in mice [7]. In this study, silibinin-induced autophagy exerted an anti-apoptosis effect. We also found there was a positive feedback loop between ROS and autophagy. This mechanism enhanced the brain-protective effect of autophagy induced by silibinin.

PI3K family members include three types. Many studies reported that type I PI3K was a kind of autophagic inhibitor [48–51] and the inhibitory effect relied on activation of Akt [52] or partly relied on activation of mTOR [50]. Type III PI3K, usually considered as an autophagic enhancer, plays an important role in the formation of autophagosome in mammalian cells. Therefore, wortmannin, the specific PI3K family inhibitor, may exert a complex effect on autophagy. In this study, wortmannin not only inhibited the autophagic effect, but also decreased NF-κB transposition to block the autophagic effect. The P38/NF-κB pathway was in a traditional way involved in AD, but silibinin did not affect the activities of P38 and JNK.

Ras/Raf/MEK/ERK and PI3K/NF-κB pathways are both essential in mediating cell proliferation, differentiation, development and transformation. However, in most reports, there seems no direct relationship between two pathways [53,54], although in some studies these pathways have the same upstream regulator such as EGF receptor, HER-2 receptor [55] and IL-3 receptor [56,57]. In a few studies, Ras is an upstream regulator of PI3K and MEK/ERK [58–60], suggesting that Ki-Ras preferentially activates Raf/MEK/ERK, but another member Ha-Ras preferentially activates the PI3K-Akt pathway [61–63]. There results coincide with ours.

In this work, Western blot analysis showed that ROS was upstream of Ras. Therefore, silibinin-induced ROS exerted a brain-protective effect through Ras/Raf/MEK/ERK and Ras/PI3K/NF-κB pathways. Some studies found that a selective MEK inhibitor, U0126, blocked the ROS-induced phosphorylation of ERK1/2 in PC12 cells and increased the levels of active caspase-3 in Ras dominant-negative mutant cells [64]. Another study found that when human U937 cells grown in 10% serum were exposed to serum-free media, the cells resulted in apoptosis and the ROS/PI3K interaction also induced a NF-κB-dependent survival pathway [65].

In conclusion, there are two pathways downstream of ROS. One is the Ras/Raf/MEK/ERK pathway in which an anti-apoptosis effect is directly exerted by bypassing autophagy. Another is the Ras/PI3K/NF-κB pathway induced autophagy which exerts an anti-apoptosis effect. Silibinin induces a protective effect by provoking ROS generation, especially •OH, and its two downstream pathways, therefore protecting PC12 cells from SNP-induced cell insult. The present study might provide a basic view for the further study on neuron system diseases.

Acknowledgements

This work was supported by the National Key Scientific Project for New Drug Discovery and Development, (2009ZX09301-012), 2009-2010, P. R. China.

Declaration of interest

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

References

[1] Pradhan SC, Girish C. Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine. Indian J Med Res 2006;124:491–504.

[2] Zhou B, Wu LJ, Li LH, Tashiro S, Onodera S, Uchiumi F, et al. Silibinin protects against Isoproterenol-induced rat cardiac myocyte injury through mitochondrial pathway after up-regulation of SIRT1. J Pharmacol Sci 2006;102:387–395.

[3] Wang MJ, Lin WW, Chen HL, Chang YH, Ou HC, Kuo JS, et al. Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. Eur J Neurosci 2002;16:2103–2112.

[4] Lu P, Mamiya T, Lu LL, Mouri A, Niwa M, Kim H-C, et al. Silibinin attenuates cognitive deficits and decreases of dopamine and serotonin induced by repeated methamphetamine treatment. Behav Brain Res 2010;207:387–393.

[5] Kidd P, Head K. Review of the bioavailability and clinical efficacy of milk thistle phytosome: a silybin-phosphatidylcholine complex (Siliphos). Altern Med Rev 2005;10:193–203.

[6] Wu J-W, Lin L-C, Hung S-C, Lin C-H, Chi C-W, Tsai T-H. Hepatobiliary excretion of silibinin in normal and liver cirrhotic rats. Am Soc Pharmacol Exp Ther 2008;36: 589–596.
[7] Wang Q, Zou L, Liu W, Hao W, Tashiro S, Onodera S, Inkejima T. Inhibiting NF-κB activation and ROS production are involved in the mechanism of silibinin's protection against D-galactose-induced senescence. Pharmacol Biochem Behav 2011;98:140–149.
[8] Rui YC, Zhang DZ, Sun DX, Zeng GQ. Effects of silybin on production of oxygen free radical, lipoperoxide and leukotrienes in brain following ischemia and reperfusion. Zhongguo Yao Li Xue Bao 1990;11:418–421.
[9] Westerink RHS, Ewing AG. The PC12 cell as model for neurosecretion. Acta Physiol (Oxf) 2008;192:273–285.
[10] Liu S, Han Y, Zhang T, Yang Z. Protective effect of trifluoperazine on hydrogen peroxide-inducedapoptosis in PC12 cells. Brain Res Bull 2011;84:183–188.
[11] Li X, Ye X, Li X, Sun X, Liang Q, Tao L, et al. Salidroside protects against MPP^+-induced apoptosis in PC12cells by inhibiting the NO pathway. Brain Res 2011; Epub ahead of print.
[12] Tan B, Luan Z, Wei X, He Y, Wei G, Johnstone BH, et al. AMPK mediates adipose stem cell-stimulated neuritogenesis of PC12 cells. Neuroscience 2011; Epub ahead of print.
[13] Mittar D, Sehajpal PK, Lander HM. Nitric oxide activates Rap1 and Ral in a Ras-independent manner. Biochem Biophys Res Commun 2004;322:203–209.
[14] Bolanos JP, Almeida A, Stewart V, Peuchen S, Land JM, Clark JB, Heales SJ. Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. J Neurochem 1997;68:2227–2240.
[15] Togo T, Katsuse O, Iseki E. Nitric oxide pathways in Alzheimer's disease and other neurodegenerative dementias. Neurol Res 2004;26:563–600.
[16] Kühn K, Lotz M. Mechanisms of sodium nitroprusside-induced death in human chondrocytes. Rheumatol Int 2003; 23:241–247.
[17] Gui J, Song Y, Reena Han N-L, Sheu F-S. Characterization of transcriptional regulation of neurogranin by nitric oxide and the role of neurogranin in SNP-induced cell death: implication of neurogranin in an increased neuronal susceptibility to oxidative stress. Int J Biol Sci 2007;3:212–224.
[18] Pytlowany M, Strosznajder JN, Jęśko H, Cąkała M, Strosznajder RP. Molecular mechanism of PC12 cell death evoked by sodiumnitroprusside, a nitric oxide donor. Acta Biochim Polonica 2008;55:339–347.
[19] Bartosz G. Reactive oxygen species: destroyers or messengers. Biochem Pharmacol 2009;77:1303–1315.
[20] Fenton H.J.H. Oxidation of tartaric acid in presence of iron. J Chem Soc Trans 1894;65:899–911.
[21] Oravecz K, Bazsó-Dombi E, Jeney F, Nagy K, Gecse M, Zs.-Nagy I. The involvement of hydroxyl free radicals in differentiation of the PC-12 rat pheochromocytoma cell line. Arch Gerontol Geriatr 2001;33:61–69.
[22] Lockshin RA, Zakeri Z. Apoptosis, autophagy, and more. Int J Biochem Cell Biol 2004;36:2405–2419.
[23] Levine B. Autophagy and cancer. Nature 2007;446:745–747.
[24] Mariño G, López-Otín C. Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cell Mol Life Sci 2004;61:1439–1454.
[25] Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. Neuroscience 2009;164:541–551.
[26] Wang Y, Singh R, Massey AC, Kane SS, Kaushik S, Grant T, et al. Loss of macroautophagy promotes or prevents fibroblast apoptosis depending on the death stimulus. J Biol Chem 2008;283:4766–4777.
[27] Choi YT, Jung CH, Lee SR, Bae JH, Baek WK, Suh MH, et al. The green tea polyphenol (-)-epigallocatechin gallate attenuates–amyloid-induced neurotoxicity in cultured hippocampal neurons. Life Sci 2001;70:603–614.
[28] Nalini K, Rayudu G, Usha G, Jinah C, Henry JF. Role of protein kinase C in basal and hydrogen peroxide-stimulated NF-κB activation in the murine macrophage J774A.1 Cell line. Arch Biochem Biophys 1998;350:206–221.
[29] Rizzardini M, Lupi M, Bernasconi S, Mangolini A, Cantoni L. Mitochondrial dysfunction and death in motor neurons exposed to the glutathione-depleting agent ethacrynic acid. J Neurol Sci 2003;207:51–58.
[30] Rego AC, Vesce S, Nicholls DG. The mechanism of mitochondrial membrane potential retention following release of cytochrome c in apoptotic GT1-7 neural cells. Cell Death Diff 2001;8:995–1003.
[31] Pocernich CB, La Fontaine M, Butterfield DA. *In-vivo* glutathione elevation protects against hydroxyl free radical-induced protein oxidation in rat brain. Neurochem Int 2000;36:185–191.
[32] Ross D. Glutathione, free radicals and chemotherapeutic agents. Mechanisms of free-radical induced toxicity and glutathione-dependent protection. Pharmacol Ther 1988;37: 231–249.
[33] Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin-phosphatidylinositol3-kinase complex functions at the trans-Golgi network. EMBO Rep 2001;2:330–335.
[34] Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000;19:5720–5728.
[35] Palmer AM. Pharmacotherapy for Alzheimer's disease: progress and prospects. Trends Pharmacol Sci 2002;23:426–433.
[36] Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet 2006;368:387–403.
[37] Alkam T, Nitta A, Mizoguchi H, Saito K, Seshima M, Itoh A, et al. Restraining tumor necrosis factor-alpha by thalidomide prevents the Amyloid b-induced impairment of recognition memory in mice. Behav Brain Res 2008;189:100–106.
[38] Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci 1997;17:2653–2657.
[39] Lichtenstein MP, Carriba P, Masgrau R, Pujol A, Galea Staging E. Anti-inflammatory therapy in alzheimer's disease. Front Ageing Neurosci 2010;2:142–142.
[40] Lu P, Mamiya T, Lu LL, Mouri A, Niwa M, Hiramatsu M, et al. Silibinin attenuates amyloid $\beta_{25\text{-}35}$peptide-induced memory impairments: implication of inducible nitric-oxide synthase and tumor necrosis factor-α in mice. JPET 2009;331: 319–326.
[41] Hou YC, Liou KT, Chern CM, Wang YH, Liao JF, Chang S, et al. Preventive effect of silymarin in cerebral ischemia-reperfusion-induced brain injury in rats possibly through impairing NF-κB and STAT-1 activation. Phytomedicine 2010;17:963–973.
[42] Bannwart CF, Peraçoli JC, Nakaira-Takahagi E, Peraçoli MT. Inhibitory effect of silibinin on tumour necrosis factor-alpha and hydrogen peroxide production by human monocytes. Nat Prod Res 2010;24:1747–1757.
[43] Duan WJ, Li QS, Xia MY, Tashiro S, Onodera S, Ikejima T Silibinin. Activated p53 and induced autophagic death in human fibrosarcoma HT1080 cells via reactive oxygen species-p38 and c-Jun N-Terminal kinase pathways. Biol Pharm Bull 2011;34:47–53.
[44] Wang HJ, Wei XF, Jiang YY, Huang H, Yang Y, Fan SM, ET AL. Silibinin induces the generation of nitric oxide in human breast cancer MCF-7 cells. Free Radic Res 2010;44:577–584.
[45] Nagy K, Pa´sti G, Bene L, Zs.-Nagy I. Induction of granulocytic maturation of HL-60 human leukemia cells by free

radicals. A hypothesis of cell differentiation involving hydroxyl radicals. Free Rad Res Commun 1993;19:10–15.
[46] Nagy K, Pa´sti G, Bene L, Zs.-Nagy I. Involvement of Fenton reaction products in differentiation induction of K562 human leukemia cells. Leukemia Res1995;19:203–212.
[47] Bishop NA, Liu T, Yankner BA. Neural mechanisms of ageing and cognitive decline. Nature 2010;464125:529–535.
[48] Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, et al. The tumor suppressor PTEN positively regulates macro-autophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 2001; 276:35243–35246.
[49] Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098–1101.
[50] Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 2005;5:726–734.
[51] Lefranc F, Kiss R. Autophagy, the Trojan horse to combat glioblastomas. Neurosurg Focus 2006;20:E7.
[52] Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098–1101.
[53] Schmitz KJ, Wohlschlaeger J, Alakus H, Bohr J, Stauder MA, Worm K, et al. Activation of extracellular regulated kinases (ERK1/2) but not AKT predicts poor prognosis in colorectal carcinoma and is associated with k-ras mutations. Virchows Arch 2007;450:151–159.
[54] Yamamoto S, Tomita Y, Hoshida Y, Morooka T, Nagano H, Dono K, et al. Prognostic signifi cance of activated Akt expression in pancreatic ductal adenocarcinoma. Clin Cancer Res 2004;10:2846–2850.
[55] Settakorn J, Kaewpila N, Burns GF, Leong AS. FAT, E-cadherin, beta catenin, HER 2/neu; Ki67 immuno-expression, and histological grade in intrahepatic cholangiocarcinoma. J Clin Pathol 2005;59:1249–1254.
[56] Watanabe S, Itoh T, Arai K. JAK2 is essential for activation of c-fos and c-myc promoters and cell proliferation through the human granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells. J Biol Chem 1996;271: 12681–12686.
[57] Tian SS, Tapley P, Sincich C, Stein RB, Rosen J, Lamb P. Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of JAKs, STAT5, and/or STAT3 are required for regulation of three distinct classes of immediate early genes. Blood 1996;88:4435–4444.
[58] Chang F, Lee JT, Navolanic PM, Steelman JG, Blalock WL, Franklin RA, et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia 2003;17: 590–603.
[59] Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PN, Blalock WL, et al. Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. Int J Oncol 2003;22:469-480.
[60] Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, et al. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia 2003;17:1263–1293.
[61] Yan J, Roy S, Apolloni A, Lane A, Hancock JF. Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. J Biol Chem 1998;273:24052–24056.
[62] Matallanas D, Arozarena I, Berciano MT, Aaronson DS, Pellicer A, Lafarga M, et al. Differences on the inhibitory specificities of HRas, K-Ras and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization. J Biol Chem 2003;278:4572–4581.
[63] Pells S, Divjak M, Romanowski P, Impey H, Hawkins NJ, Clarke AR, et al. Developmentally-regulated expression of murine K-ras isoforms. Oncogene 1997;15:781–786.
[64] Jiang H, Zhang L, Koubi D, Kuo J, Groc L, Rodriguez AI, et al. Roles of Ras-Erk in apoptosis of PC12 cells induced by trophic factor withdrawal or oxidative stress. J Mol Neurosci 2005;25:133–140.
[65] Lee SB, Hong SH, Kim H, Um HD. Co-induction of cell death and survival pathways by phosphoinositide 3-kinase. Life Sci 2005;78:81–91.

This paper was first published online on Early Online on 13 May 2011.