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ORIGINAL PAPER

Neuroprotective Effects of Ugonin K on Hydrogen Peroxide-Induced Cell Death in Human Neuroblastoma SH-SY5Y Cells

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Abstract Oxidative stress plays an important role in the pathological processes of various neurodegenerative diseases. Ugonin K, a flavonoid isolated from the rhizomes of *Helminthostachys zeylanica*, possesses potent antioxidant property. In this study, we investigate the neuroprotective effects of ugonin K on hydrogen peroxide (H_2O_2)-induced apoptosis in SH-SY5Y cells. Incubation of SH-SY5Y cells with H_2O_2 for 24 h induced cell death measured with MTT assay. Hoechst 33258 staining confirmed that the reduced cell viability by H_2O_2 was due to apoptosis. In addition, H_2O_2 increased the expression of 17-kDa cleaved fragment of caspase-3 which could be reversed by pretreatment with ugonin K. Pretreatment with ugonin K attenuated H_2O_2-induced cell death in a dose-dependent manner. Neuroprotective effect of ugonin K was abolished by ERK and PI3K inhibitors. Pretreatment with JNK kinase and p38 MAPK inhibitors had no effect on ugonin K-mediated protection against H_2O_2-induced apoptosis. Western blotting with anti-phospho-ERK1/2 and anti-phospho-Akt (pS473) antibodies showed that ugonin K increased both ERK1/2 and Akt phosphorylation. These results suggest that ugonin K by activation of ERK1/2 and PI3K/Akt signal pathways protects SH-SY5Y cells from H_2O_2-induced apoptosis.

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Introduction

Oxidative stress has long been implicated both in the physiological process of aging and in a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and ischemic injury [1, 2]. Oxidative stress mediated by reactive oxygen species (ROS) was generated following cell lysis, oxidative burst or the presence of an excess of free transition metals [3]. In many cells, ROS caused DNA damage, oxidative of proteins, peroxidation of lipids, and thus induced apoptosis [4]. Therefore, therapeutic strategies aimed at preventing or delaying ROS production might be a reasonable choice for the treatment of these neurodegenerative diseases.

Flavonoids are naturally occurring polyphenolic compounds presented in a variety of fruit, vegetables, and seeds [5]. Flavonoids have many biological and pharmacological activities including antioxidative, anti-inflammatory, antiviral, and antitumor effects [6]. Recent studies demonstrated that both antioxidative and anti-inflammatory properties of flavonoids contributed to their neuroprotective effects [7–9]. Ugonin K was isolated from the rhizomes of *Helminthostachys zeylanica* (L.) Hook. The rhizomes of *H. zeylanica*, named "Daodi-Ugon" is a Chinese herbal medicine used as an antipyretic and antiphlogistic agent. In the 1,1-dipheny-2-picrylhydrazyl (DPPH) assay, Ugonin K was found to have potent antioxidative activity [10]. Understanding the molecular basis of neuroprotective actions of flavonids would provide important clues for drug design as neuroprotectants. The purpose of this study is to evaluate whether ugonin K

protects SH-SY5Y neuroblastoma cells from H_2O_2-induced neurotoxicity. We also wish to unravel the mechanism behind ugonin K's neuroprotective action.

Experimental Procedure

Chemicals and Reagents

Ugonin K (purity >95%), provided by the Department of Phytochemistry at this Institute. Hydrogen peroxide (H_2O_2) was purchased from Calbiochem (Merck Ltd. Taiwan). MTT, Hoechst 33258, U0126, PD98059, Wortmannin, LY294002, SP600125, SB202190, and SB203580 were obtained from Sigma (St. Louis, MO). Phospho-ERK, ERK, phospho-Akt, Akt antibodies were purchased from Cell Signaling Tech (Beverly, MA). β-actin antibodies was purchased from Santa Cruz Biotech. (Santa Cruz, CA). Goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson (West Grove, PA).

Cell Culture

The human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (ATCC). Cells were seeded into plates or dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37°C. Cells were plated at a density of 5×10^4 cells per well in 96-well plates for cell viability assay, 1×10^6 cells per well in 6-well with coverslips for Hoechst staining, 1×10^7 cells per plate in 90 mm dishes for western blotting and allowed to adhere 24 h before use. In order to produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. Cells were pre-treated with ugonin K for 1 h before the addition of H_2O_2 and 24 h later the survival of neurons was determined by MTT assays.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

SH-SY5Y cells were plated at a density 5×10^4 cells per well in 96 well plates, and cell survival was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays. The reduction status of the cells was measured by a colorimetric assay for cell survival [11]. MTT was dissolves in phosphate-buffered saline (PBS, 500 ml contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) at a concentration of 5 mg/ml and filtered (Millipore). After 24 h exposure to H_2O_2, 10 μl of MTT were added to each well and incubated for 2 h at 37°C in the dark. When taken up by living cells, MTT is converted to a water-insoluble blue product (formazan). The formazan product was dissolved by adding 150 μl dimethylsulfoxide (DMSO) to each well. The absorption value at wavelength of 570 nm was determined with an ELISA plate reader. Data were presents as the percentage of survival relative to vehicle-treated control culture. All measurements were performed in triplicate and each experiment was repeated at least three times.

Hoechst 33258 Staining

Nuclei were stained with Hoechst 33258 (bis-benzimide, Sigma) to detect chromatin condensation or nuclear fragmentation characteristic of apoptosis. After 12 h H_2O_2 treatment, cells were stained with 5 μg/ml Hoechst 33258 for 10 min. After being washed with PBS twice, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at 25°C. Fluorescence of the soluble DNA (apoptotic) fragments was measured in a Varian Fluorometer at excitation wavelength of 365 nm and emission wavelength 460 nm.

Western Blot Assay

The treated cells (1×10^7 cells/10 ml in 90 mm dish) were collected and washed with PBS. After centrifugation, cells were lysed in a lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and 100 lg/ml leupeptin. The lysates were incubated on ice for 30 min and centrifuged at 12,000g for 20 min. Supernatants were collected and followed by protein concentration determination using Bradford Assay. After addition of 5× sample loading buffer containing 625 mM Tris–HCl, pH 6.8, 500 mM dithiothreitol, 10% SDS, 0.06% bromophenol blue, 50% glycerol, protein samples were electrophoresed on 8% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was incubated in blocking buffer (Tris–buffered saline, pH 7.4, containing 5% nonfat dry milk) on ice for 60 min, reacted with anti-ERK (1:1000), anti-Akt (1:1000), ant-phospho-ERK (1:1000), anti-phospho-Akt (1:1000), anti-cleaved caspase-3 (1:1000)and anti-β-actin (1:1000) antibodies for overnight at 4°C. The membrane was washed three times for 5 min each using TBST (TBS and 0.05% Tween 20). After that it was incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5000) for 1 h at room temperature and washed again three times in TBST buffer. Immunoreactivity was detected by using the western blot chemiluminescence reagent system (PerkinElmer, Boston, MA). The membrane was incubated with ECL substrate solution for 5 min. Films were exposed at different time points to ensure the optimum density, but not saturated.

Statistical Analysis

A single-factor analysis of variance (ANOVA) and post hoc comparisons were used to analyze the concentration-dependent effects of (1) H_2O_2 on cell viability; (2) the effect of ugonin K on H_2O_2-induced cell death and (3) H_2O_2-induced activation of caspase-3. Student's *t*-test was used to compare the significant difference between control and drug-treated groups. All values are presented as mean ± standard error of the mean. Levels of $P < 0.05$ were considered to be of statistical significance.

Results

H_2O_2-Induced Cytotoxicity in SH-SY5Y Cells

SH-SY5Y cells were exposed to H_2O_2 (40–200 μM) for 24 h and cell viability was assessed by MTT metabolism assays. As shown in Fig. 1A, H_2O_2 decreased cell viability in a dose-dependent manner. Exposure of 40, 60, 80, 100, 150, and 200 μM of H_2O_2 reduced the survival to 90.4 ± 1.8, 69.2 ± 0.9, 52.7 ± 1.5, 34.9 ± 2.7, 23.1 ± 1.7, and 17.8 ± 0.9% of control respectively ($F_{(6,28)} = 1224$, $P < 0.001$), $n = 5$ experiments in each concentration). Time course studies were performed to determine the temporal induction of cell death by H_2O_2. Viability decreased significantly 3 h after the incubation with 100 μM of H_2O_2 and continued to decrease over the next 24 h ($F_{(4,20)} = 679.5$, $P < 0.0001$) (Fig. 1B).

Ugonin K Prevented SH-SY5Y Cells Against H_2O_2-Induced Cell Death

The effect of ugonin K on H_2O_2-induced cell death was examined in SH-SY5Y cells. As illustrated in Fig. 1C, H_2O_2 (100 μM for 24 h) treatment decreased cell survival to 35.9 ± 2.7%. Pre-treatment with ugonin K (1, 2.5, 5, 10, 20 μM) prevented cell death, restoring cell survival to 50.2 ± 2.7, 59.0 ± 2.0, 68.7 ± 2.8, 81.9 ± 1.7 and

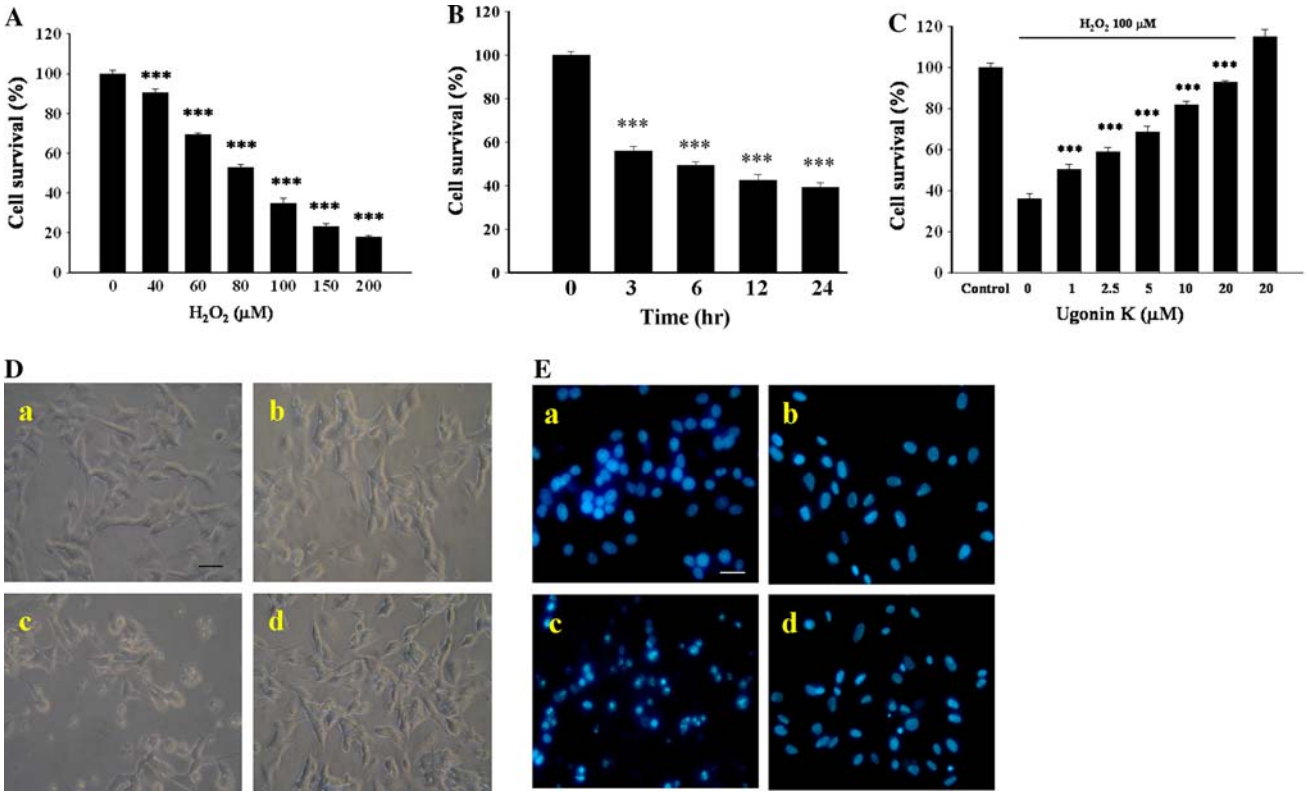

Fig. 1 Neuroprotective effects of ugonin K against H_2O_2 toxicity in SH-SY5Y cells. **A** Concentration-dependent effect of H_2O_2 on cell survival in SH-SY5Y cells. SH-SY5Y cells were exposed to different concentrations of H_2O_2 for 24 h. Cell viability was assessed using MTT assay. Cells without treatment serve as control. *** $P < 0.001$ vs. Control. **B** Time course of cell death induced by 100 μM of H_2O_2. Viability decreased significantly 3 h after the incubation with H_2O_2 and continued to decrease over the next 24 h. **C** Neuroprotective effects of ugonin K on H_2O_2 -induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were treated with 100 μM H_2O_2 for 24 h. Some cells were pretreated with 1, 2.5, 5, 10, and 20 μM ugonin K for 1 h prior to incubation with 100 μM H_2O_2 for an additional 24 h. *** $P < 0.001$ vs. H_2O_2 alone. (**D** and **E**) SH-SY5Y cells were treated with 20 μM ugonin K (**b**), 100 μM H_2O_2 (**c**), or both (**d**) for 24 h in C and 12 h in D. Morphological studies were conducted by phase-contrast microscopy (**D**) or Hoechst 33258 staining (**E**). Control cells were without any treatment (**a**). The arrowheads indicate apoptotic nuclei. Scale bar = 50 μm

92.8 ± 0.6%, respectively ($F_{(5,24)} = 411.3$, $P < 0.0001$). Ugonin K at 20 μM alone did not cause any apparent neurotoxicity, but cell viability was significantly increased from 100% to 115%. These results suggest that Ugonin K induce cell proliferation is involved in protection of SH-SY5Y cells.

The neuroprotective effect of ugonin K was confirmed using the morphological analysis (Fig. 1C). H_2O_2-treated neurons exhibited the disappearance of cellular processes, the decrease of refraction and the falling to pieces which could be prevented by ugonin K. To determine whether the reduced cell viability was due to apoptosis, SH-SY5Y cells were stained with Hoechst 33258. As shown in Fig. 1D, control cells without treating H_2O_2 exhibited uniformly dispersed chromatin, normal organelle and intact cell membrane. Cells treated with 100 μM H_2O_2 for 12 h showed typical characteristics of apoptosis including the condensation of chromatin, the shrinkage of nuclear and the appearance of apoptotic bodies. However, the number of cells with nuclear condensation and fragmentation was markedly decreased when the cells were pre-treated 20 μM ugonin K.

Ugonin K Prevents H_2O_2-Induced Activation of Caspase-3

Caspases are key mediators of cell death and caspase-3 is an executioner for the death program in cortical neurons in response to various noxious insults. We examined whether H_2O_2-induced cell death was dependent on caspase-3 activation. Constitutive expression of the 32-kDa pro-caspase-3 protein was detected in controls. After treatment with H_2O_2, there was a concentration-dependent effect of H_2O_2 on the expression of 17-kDa cleaved fragment of caspase-3 ($F_{(3,8)} = 116.5$, $P < 0.001$) (Fig. 2a). Furthermore, the effect of H_2O_2 could be reversed by pretreatment with ugonin K (Fig. 2b).

Ugonin K-Mediated Protective Action Involves Extracellular Signal Regulated Kinase 1 and 2 (ERK1/2) Activation

In the nervous system, ERK1/2 is critical for neuronal differentiation, plasticity, and survival [12]. ERK1/2 mediates neuroprotective activity of extracellular factors including neurotrophins [13–15]. To see if ERK pathway was involved in the action of ugonin K, we pre-treated cells with ugonin K in the presence of ERK1/2 inhibitors, U0126 and PD98059. Pre-preatment with U0126 or PD98059 abolished ugonin K-mediated protection against H_2O_2 -induced apoptosis (Fig. 3). Cell viability was significantly decreased from 92.8% to 68.3%, 50.8% and 43.1% ($F_{(3,16)} = 1338$, $P < 0.0001$) with 25, 50, and 100 μM

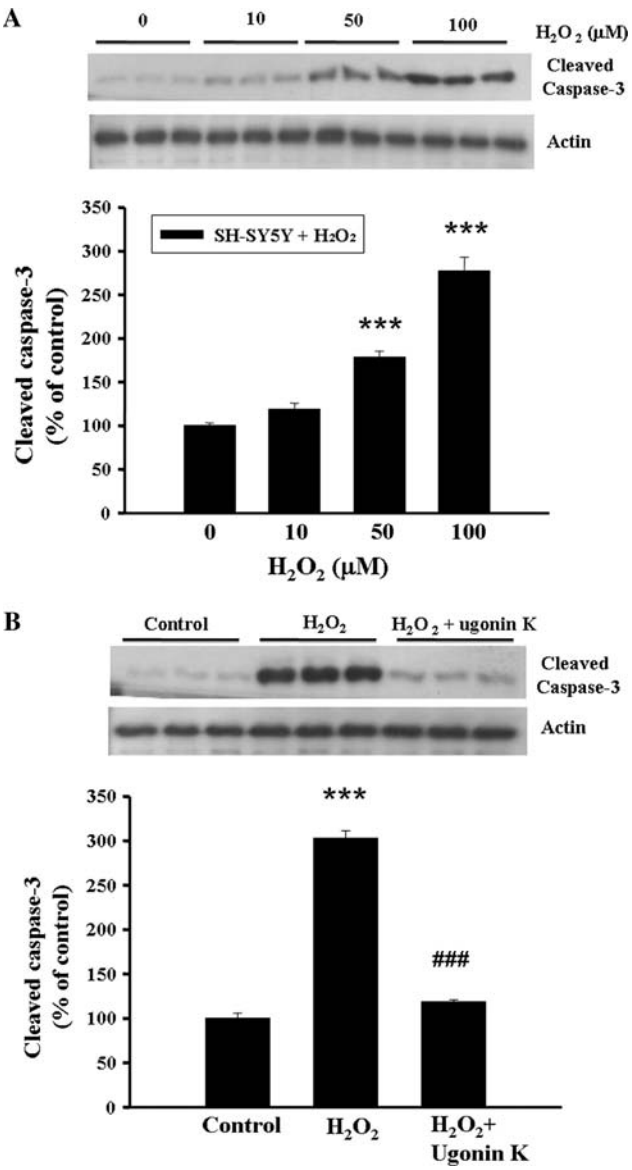

Fig. 2 Effects of ugonin K on H_2O_2-induced expression of cleaved caspase-3. **a** Effect of H_2O_2 on caspase-3 protein expression in SH-SY5Y cells. Representative Western blot showing 32- and 17-kDa (cleaved) bands of caspase-3. The pro-form caspase-3 is constitutively present in the SH-SY5Y cells. Exposure of cells to H_2O_2 progressively increased p17 cleaved fragment of caspase-3. *** $P < 0.001$ vs. vehicle control. **b** Exposure of cells to H_2O_2 (100 μM) increased expression of p17 cleaved fragment of caspase-3. The increase in cleaved caspase-3 was reversed by 20 μM ugonin K. *** $P < 0.001$ vs. vehicle control. $^{###}$ $P < 0.001$ vs. H_2O_2

U0126 and from 92.8% to 69.3%, 67.1% and 61.0% ($F_{(3,16)} = 289.3$, $P < 0.001$) with 25, 50, and 100 μM PD98059. These results suggest that ERK pathway is involved in ugonin K-mediated protection of SH-SY5Y cells against H_2O_2 -induced cell death.

We monitored ERK1/2 activity during ugonin K-mediated protective actions by western blotting using a phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}) antibody.

Figure 3c shows that application of ugonin K (20 μM) significantly increased phosphorylation of ERK1/2 at 20 min after application, peaked around 60 min and lasted for 3 h. Next, we determined the concentration-dependent effect of ugonin K on ERK1/2 phosphorylation (Fig. 3d). These results are consistent with the notion that ugonin K-mediated protective action involves ERK1/2 activation.

Ugonin K-Mediated Protective Action Involves PI3K-Dependent Akt Activation

PI3K and its downstream effector Akt has been shown to suppress apoptosis and promote cell survival [13, 14, 16]. To see if PI3K pathway was involved in the action of ugonin K, we pre-treated cells with ugonin K in the presence of PI3K inhibitors, wortmannin, and LY294002. Pretreatment with wortmannin or LY294002 abolished ugonin K-mediated protection against H_2O_2 -induced apoptosis (Fig. 4). Cell viability was significantly decreased from 92.8% to 64.6%, 62.8% and 59.5% ($F_{(3,16)} = 416.3$, $P < 0.001$) with 25, 50, and 100 nM wortmannin pretreatment and from 92.8% to 66.1%, 60.0% and 57.4% ($F_{(3,16)} = 382.8$, $P < 0.001$) with 0.5, 1, and 5 μM LY294002 pretreatment. These results suggest that PI3K pathway is involved in ugonin K-mediated protection of SH-SY5Y cells against H_2O_2 -induced cell death.

Since phosphorylation of Akt at Ser^{473} is required for its full activation, we monitored PI3K activity during ugonin K-mediated protective actions by western blotting using a phoso-specific Akt at Ser^{473} antibody. Figure 4c shows that application of ugonin K significantly increased phosphorylation of Akt at 10 min after application, peaked at 20 min and lasted for at least 30 min. These results are consistent with the notion that ugonin K-mediated protective action involves Akt activation.

JNK1/2 and p38 are not Involved in SH-SY5Y Cells Protective Against H_2O_2-Induced Apoptosis

ERKs, c-Jun N-terminal kinase (JNK) and p38 kinase are the family of mitogen-activated protein kinases (MAPK). JNK and p38 are widely distributed in mammalian tissue including the brain [17]. JNKs and their substrates like the transcription factor c-Jun are linked to both neurodegeneration and neuroprotection [18]. To investigate whether JNK and p38 MAPK pathways were involved in the action of ugonin K, we pre-treated cells with ugonin K in the presence of JNK inhibitor SP600125 and p38 MAPK

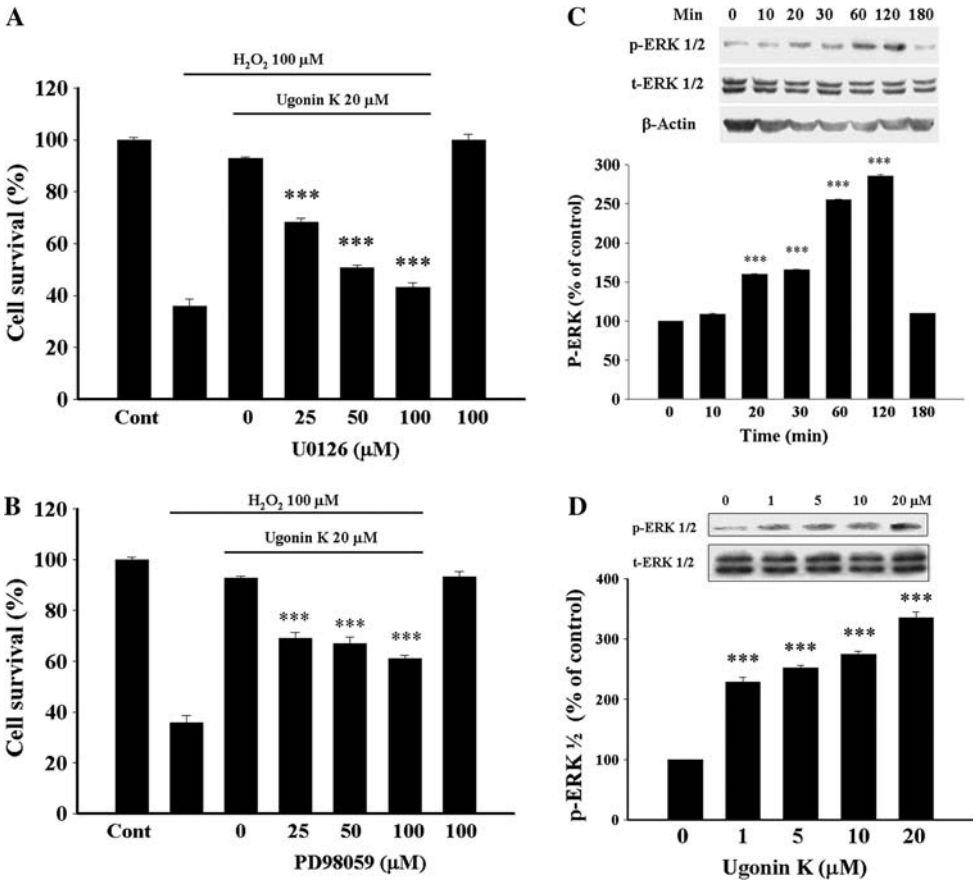

Fig. 3 Effect of ERK 1/2 inhibitors on neuroprotective effect of ugonin K. Application of different concentrations of U0126 (**a**) or PD98059 (**b**) attenuated the neuroprotective effect of ugonin K in a concentration-dependent manner. *** $P < 0.001$ vs. H_2O_2 + ugonin K. (**c** and **d**) Ugonin K-mediated protective action is associated with activation of ERK1/2. Cells were treated with 20 μM ugonin K for various time points as indicated **c** or were treated with different concentrations of ugonin K for 60 min **d**. Protein samples were separated by SDS-PAGE and subjected to immunoblotting with antibodies specific to phospho-ERK1/2 (p-ERK1/2), ERK1/2, and β-actin (n = 3 independent experiments). *** $P < 0.001$ vs. control

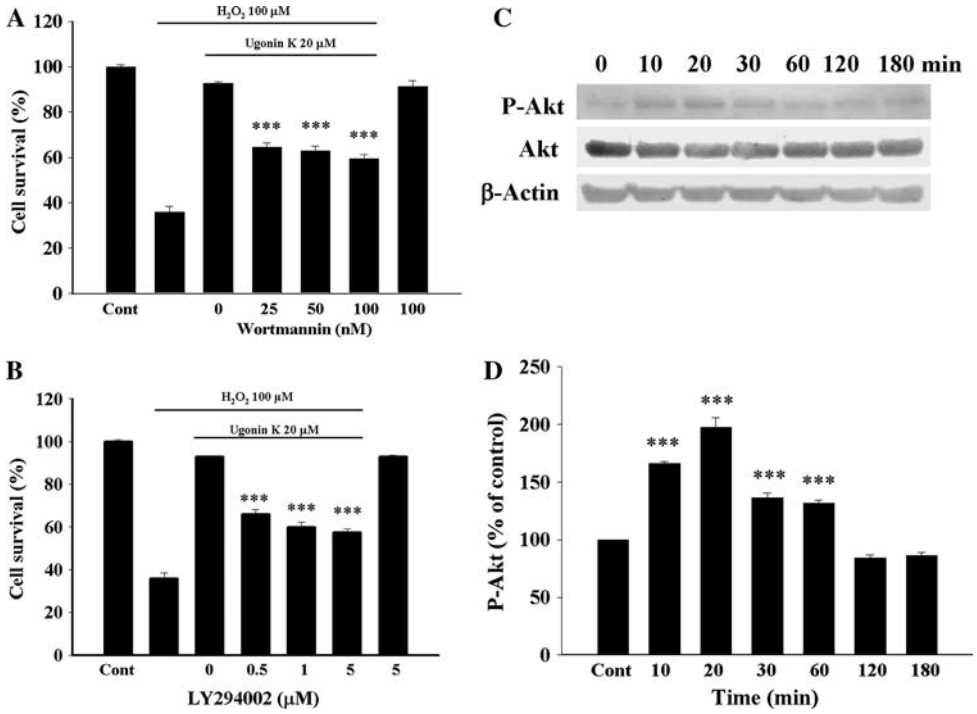

Fig. 4 Effect of PI3K inhibitors on neuroprotective effects of ugonin K. Application of different concentrations of wortmannin (**a**) or LY294002 (**b**) attenuated the neuroprotective effect of ugonin K. *** $P < 0.001$ vs. H_2O_2 + ugonin K. (**c** and **d**) Ugonin K-mediated protective action is associated with phosphorylation of Akt. Cells were treated with 20 μM ugonin K for various time points as indicated. Protein samples were separated by SDS-PAGE and subjected to immunoblotting with antibodies specific to phospho-Akt (p-Akt), Akt and β-actin ($n = 3$ independent experiments). *** $P < 0.001$ vs. Control

inhibitors SB202190 and SB203580. Pre-preatment with SP600125 (Fig. 5), SB202190 or SB203580 (Fig. 6) had no effect on the ugonin K-mediated protection against H_2O_2 -induced apoptosis. These results suggest that JNK and p38 MAPK pathways are not involved in ugonin K-mediated protection of SH-SY5Y cells against H_2O_2 -induced cell death. As shown in Figs. 5 and 6, SP600125, SB202190, and SB203580 had no significant effect on ugonin K-mediated protection of SH-SY5Y cells against H_2O_2 -induced apoptosis.

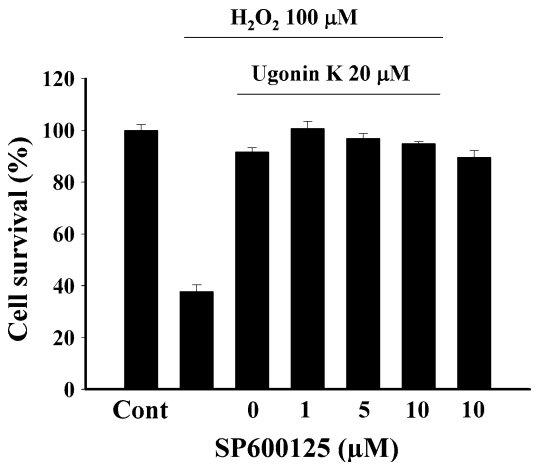

Fig. 5 JNK inhibitor fails to affect the neuroprotective effects of ugonin K. Cells well pretreated with different concentrations of SP600125 at 30 min before the addition of 20 μM ugonin K. Ugonin K was applied 1 h before the addition of 100 μM H_2O_2. Cell survival was determined by MTT assay 24 h after the addition of H_2O_2

Discussion

Oxidative stress is a major cause of cellular injuries in a variety of human neurodegenerative disorders [1, 2]. Considerable efforts have been attempted to search for natural antioxidants with neuroprotective potential. Ugonin K, a flavonoid isolated from the rhizomes of *H. zeylanica* (L.) Hook, is a potent antioxidant [10]. H_2O_2 has been extensively used as an inducer of oxidative stress [19–22]. In the present study, we found that treating SH-SY5Y cells with H_2O_2 dose-dependently and time-dependently resulted in cell death. Pretreatment of SH-SY5Y cells with different concentrations of ugonin K decreased cell death in a dose-dependent manner and the result was further confirmed by morphological observation. These results indicate that ugonin K is able to protect SH-SY5Y cells from H_2O_2 -induced neurotoxicity. Furthermore, when stained with fluorescent DNA binding dye Hoechst 33258, H_2O_2 -treated cells displayed typical morphological features of apoptosis with sickle shaped-nuclei. Ugonin K pretreatment mitigated these morphological changes. In addition, H_2O_2 increased the expression of 17-kDa cleaved fragment of caspase-3 which could be reversed by pretreatment with ugonin K. It was noted that Ugonin K at 20 μM alone (in the absence of H_2O_2) significantly increased cell number to 115% of control. These data suggest that ugonin K-induced cell proliferation also contributes to its neuroprotective effect.

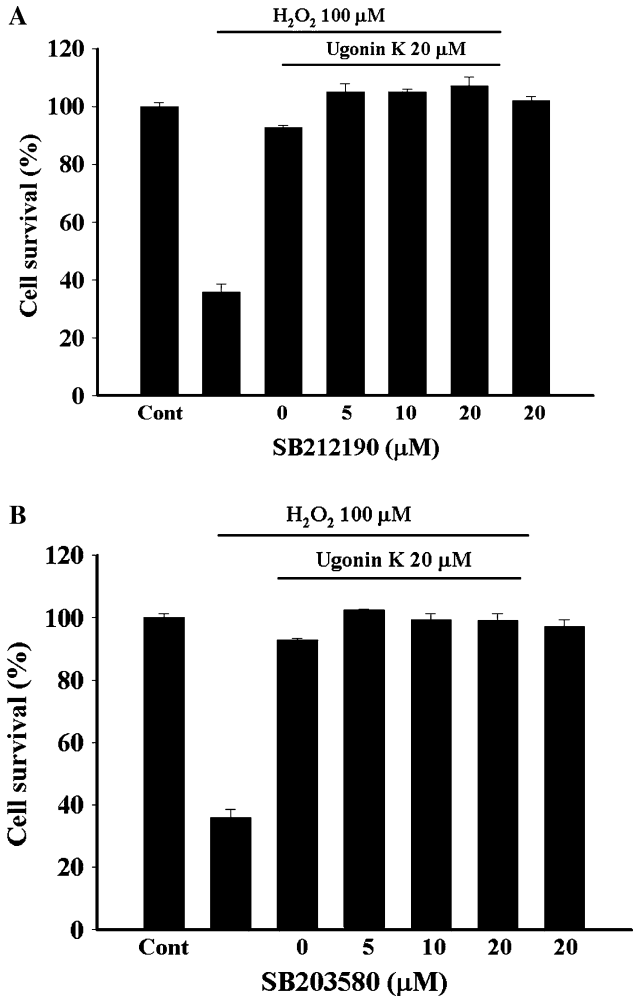

Fig. 6 p38 MAPK inhibitors fail to affect the neuroprotective effect of ugonin K. Cells were pretreated with different concentrations of SB202190 (**a**) or SB203580 (**b**) at 30 min before the addition of 20 μM ugonin K. Ugonin K was applied 1 h before the addition of 100 μM H_2O_2. Cell survival was determined by MTT assay 24 h after incubation with H_2O_2

ERK1/2 is critical for neuronal differentiation, plasticity and survival [12]. ERK1/2 mediated neuroprotective activity of extracullar factors including neurotrophins [13]. The ERK1/2 pathway affects multiple targets that stimulate production of anti-apoptotic mediators [12]. For instance, pro-survival activity of ERK1/2 in sympathetic neurons is mediated via cAMP response element binding protein (CREB)-stimulated expression of bcl-2 [23]. We found that pre-treatment with the U0126 and PD98059 completely abolished the effect of ugonin K indicating that ERK pathway is involved in ugonin K-mediated protection of SH-SY5Y cells against H_2O_2 -induced apoptosis. The hypothesis was confirmed by the observation that ERK phosphorylation was increased after application of ugonin K.

Activation of PI3-K and its downstream effecter Akt has been shown to suppress apoptosis and promote cell survival [13, 14, 16]. It has been shown that activation of PI3-K leads to phosphorylation and activation of Akt which promotes cell survival by enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic proteins [24]. The targets of Akt that have been implicated in suppression of apoptosis include the Bcl-2 family member Bad [25], caspase-9 [26], several transcription factors, and the protein kinase GSK-3β [27, 28]. In the case of transcription factor FKHRL1, it was postulated that Akt inhibited FKHRL1-mediated Fas ligand transcription thereby suppressed apoptosis [29]. We found that the effect of ugonin K was inhibited by wortmannin and LY294002 indicating that the PI3K/Akt pathway plays an important role in the anti-apoptotic effects of ugonin K in SH-SY5Y cells. In addition, Akt phosphorylation was increased after application of ugonin K. The neuroprotective effect of ugonin K was unaffected by selective inhibitors of JNK and p38 MAPK ruling out the involvement of these signal pathways in the action of ugonin K.

In summary, ugonin K could ameliorate H_2O_2-induced cell death in SH-SY5Y cells. Ugonin K protected SH-SY5Y cells from oxidative stress-induced neurotoxicity may involve ERK1/2 and PI3K/Akt pathways. Therefore, the molecular mechanisms neuroprotective effects of ugonin K may include not only their antioxidant activities but also their interaction with cell signaling cascades leading to cell survival and cell proliferation.

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