

Wogonin Induced Mitochondrial Dysfunction and Endoplasmic Reticulum Stress in Human Malignant Neuroblastoma Cells Via IRE1 α -Dependent Pathway

Wenliang Ge · Qiyu Yin · Hua Xian

Received: 28 November 2014 / Accepted: 17 February 2015 / Published online: 5 March 2015
© Springer Science+Business Media New York 2015

Abstract Wogonin, a flavonoid isolated from *Scutellaria baicalensis* Georgi, has been reported to exhibit a variety of biological effects including anti-cancer effects. It has a pro-apoptotic role in many cancer types. However, the molecular mechanisms of wogonin in treating neuroblastoma remain elusive. In the present study, two malignant neuroblastoma cell lines (SK-N-BE2 and IMR-32 cells) were treated with different doses of wogonin (0–150 μ M). Wogonin showed significant cytotoxic effects in SK-N-BE2 and IMR-32 cells in a dose- and time-dependent manner. Treatment of SK-N-BE2 and IMR-32 cells with 75 μ M wogonin for 48 h significantly promoted apoptosis, the release of cytochrome c, altered the expression of certain members of Bcl-2 family (Bcl-2, Bax and Bid), and increased the activation of caspase-3, caspase-8, caspase-9, and PARP-1, which demonstrated that the cytotoxic effect of wogonin in SK-N-BE2 and IMR-32 cells is mediated by mitochondrial dysfunction. Moreover, wogonin induced the expression of endoplasmic reticulum (ER) stress-related proteins (GRP78/Bip and GRP94/gp96) and activation of caspase-12 and caspase-4 in SK-N-BE2 and IMR-32 cells. In addition, wogonin increase the expression of IRE1 α and TRAF2, and phosphorylation of ASK1 and JNK in SK-N-BE2 and IMR-32 cells. Knockdown of IRE1 α by siRNA not only markedly inhibited wogonin-induced up-regulation of IRE1 α and TRAF2, and phosphorylation of ASK1 and JNK but also reduced wogonin-induced cytotoxic effects and mitochondrial dysfunction in SK-N-BE2 and IMR-32 cells. These results indicated that wogonin could induce apoptosis, mitochondrial dysfunction, and ER stress in SK-N-BE2 and IMR-32 cells by modulating IRE1 α -dependent pathway.

Keywords Wogonin · Malignant neuroblastoma · Apoptosis · Mitochondrial dysfunction · Endoplasmic reticulum stress · IRE1 α

Introduction

Malignant neuroblastoma is the most commonly seen extracranial childhood solid tumor derived from sympathetic nervous system that mostly affects the adrenal gland, and it often metastasizes to other body parts including the chest, neck, lymph nodes, pelvis, liver, and bone (Brodeur 2003; Irshad et al. 2004). Malignant neuroblastoma is currently being treated by surgery, radio-pharmaceutical treatment, and chemotherapy. However, the prognosis of high-risk neuroblastoma patients is usually poor (Chakrabarti et al. 2013; Schor 2009). Therefore, development of more effective chemopreventive and chemotherapeutic agents for the treatment of malignant neuroblastoma is imperative.

Wogonin is one of the major flavonoids found from the root of the Chinese herb *Scutellaria baicalensis* Georgi (also called Huang-Qin), which is widely used for the treatment of a serial of diseases for its anti-viral, anti-bacterial, anti-inflammatory, anti-oxidant, and anti-cancer effects (Gasiorowski et al. 2011; Li-Weber 2009). Previous studies both in vitro and in vivo have shown that wogonin may have anti-cancer effects in various types of cancer, such as human colorectal cancer (He et al. 2013), lung cancer (Gao et al. 2011), gallbladder carcinoma (Dong et al. 2011), breast cancer (Huang et al. 2012), hepatocellular carcinoma (Xu et al. 2013), osteosarcoma (Lin et al. 2011), and glioma cancer (Lee et al. 2012; Tsai et al. 2012).

W. Ge · Q. Yin (✉) · H. Xian (✉)
Department of Pediatric Surgery, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, People's Republic of China
e-mail: yinqiyunantong@163.com
e-mail: huaxiannantong@163.com

Apoptosis is the major mechanism for cancer cell elimination. Apoptosis can be induced through different pathways, including mitochondrial-mediated apoptotic pathway (Brenner et al. 2009; Chiantore et al. 2009; Huang et al. 2014; Xu et al. 2014) and endoplasmic reticulum (ER) stress (Boyce et al. 2006; Breckenridge et al. 2003; Hetz 2012). Previous studies also have shown that wogonin could trigger apoptosis of human osteosarcoma cells (Lin et al. 2011), hepatocellular carcinoma cells (Xu et al. 2013), gastric carcinoma (Yang et al. 2012), and glioma cancer cells (Tsai et al. 2012) through ER stress-dependent apoptotic pathways. Moreover, wogonin could elicit a potent pro-apoptotic effect in human lung cancer cells (Gao et al. 2011), breast cancer cells (Zhang et al. 2013), and myeloma cells (Huang et al. 2012) though a mitochondrial-dependent manner. Although the anti-cancer effects of wogonin have been reported in many types of carcinoma cells, its effects on malignant neuroblastoma and its action mechanisms are not known.

In this study, we investigated the molecular mechanisms of wogonin regarding its anti-tumor effect on malignant neuroblastoma cell lines (SK-N-BE2 and IMR-32 cells) by examining mitochondrial dysfunction- and ER stress-dependent apoptosis pathways. In addition, we also investigated IRE1 α -dependent pathway and its role in wogonin-induced apoptosis of SK-N-BE2 and IMR-32 cells.

Materials and Methods

Cell Culture

SK-N-BE2 and IMR-32 human malignant neuroblastoma cells were obtained from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). SK-N-BE2 cell line was propagated in RPMI 1640 (Mediatech, Manassas, VA, USA) while IMR-32 cell line was propagated in DMEM (Mediatech, Manassas, VA, USA), and both growth media were supplemented with 10 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 1 % penicillin and 1 % streptomycin (GIBCO, Grand Island, NY, USA). The cells were then allowed to grow at 37 °C in humidified 5 % CO₂, 95 % air for 24 h prior to treatment. Wogonin (3MA, Sigma Chemical, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions, and aliquots were stored at –20 °C until further used. Concentration of DMSO in all experiments was maintained at less than 0.01 % that did not affect cell growth or death.

Cell Viability Assay

Cell viability was assessed using the MTT assay. Cells were plated at a density of 10⁵ cells per well into 96-well plates in L-15 medium with 10 % fetal bovine serum. After overnight

growth, cells were exposed to different concentrations of wogonin for 48 h in 5 % CO₂ incubator at 37 °C. At the end of treatment, 20 μ l of 0.5 % MTT was added to the medium and incubated for 4 h at 37 °C. The supernatant was removed and 0.1 ml DMSO was used to dissolve precipitate. Then, formazan crystals formed by mitochondrial reduction of MTT were solubilized in DMSO and absorbance was read at 540 nm using a microplate reader (BioRad, Hercules, CA, USA).

Annexin V-FITC and Propidium Iodide Staining

Double staining for Annexin V-FITC and propidium iodide (PI) was performed to estimate the apoptotic rate of SK-N-BE2 and IMR-32 cells. Briefly, SK-N-BE2 and IMR-32 cells were treated with 75 μ M wogonin for 48 h. Subsequently, SK-N-BE2 and IMR-32 cells were trypsinized and washed twice with PBS, and centrifuged at 800 rpm for 5 min. Then, 1 \times 10⁶ cells were suspended in binding buffer and double-stained with Annexin V-FITC and PI for 30 min at room temperature. After that, the fluorescence of each sample was quantitatively analyzed by FACS calibur flow cytometer and CellQuest software. The results were interpreted as follows: PI positive and Annexin V-FITC-positive stained cells were considered in apoptosis.

Small RNA Interference (siRNA)

The siRNA constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO), the siGENOME SMARTpool IRE1 α (M-004951-01-0010). The non-targeting siRNA control, SiConTRolNon-targeting SiRNA pool (D-001206-13-20), was also obtained from Dharmacon. Cells were transfected with 50–100 nM siRNA in Opti-MEM medium (Invitrogen, Carlsbad, CA) with 5 % fetal calf serum using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's transfection protocol. Twenty-four hours after transfection, the cells were treated with or without 75 μ M wogonin for 48 h before quantitation of apoptotic cells by MTT assay and flow cytometry or the protein expression by western blot analysis. Efficiency of siRNA was measured by western blot analysis.

Western Blot Analysis

For the western blot analysis, SK-N-BE2 and IMR-32 cells were harvested, washed once in ice-cold phosphate-buffered saline, gently lysed in ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05 % digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 min, and centrifuged at 12,000 rpm at 4 °C. Protein concentration was measured using BioRad Bradford protein assay reagent and subjected to SDS-PAGE. Proteins were transferred to polyvinylidene

fluoride membranes and incubated successively in 5 % bovine serum albumin in Tris-buffered saline-Tween 20 buffer (TBST) (25 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, and 0.1 % Tween 20) for 1 h, then incubated overnight at 4 °C with specific antibodies (Table 1) followed by reaction with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) for 1 h. After each incubation, membranes were washed extensively in TBST and the immunoreactive band was detected using ECL-detecting reagents.

Cytochrome c Release Assay

After cells were incubated with 75 μ M wogonin for 48 h, SK-N-BE2 and IMR-32 cells were collected by centrifugation at 800 \times g for 5 min at 48 °C and washed with ice-cold PBS. The fractionation of the mitochondrial protein and cytosolic protein was extracted according to the instruction of Mitochondrial Protein Extraction kit (KeyGen), respectively. Cell nuclear and cytoplasmic fractions were prepared using a nuclear/cytosol fractionation kit of Biovision Inc. (Mountain View, CA) according to the manufacturer's direction. Western blot analysis was used to detect cytochrome c of cytosolic fraction and mitochondrial fraction with cytochrome c antibody.

Data Analysis

Statistical calculations of the data were performed using *t* test when two groups are compared. One-way ANOVA followed

Table 1 Specific antibodies used in the experiment

Number	Antibody name	Company	Dilution
1	Bax	Santa Cruz Biotechnology	1:500
2	Bid	Santa Cruz Biotechnology	1:500
3	Bcl-2	Santa Cruz Biotechnology	1:500
4	Cytc	Santa Cruz Biotechnology	1:500
5	Caspase-3	Cell Signaling	1:500
6	Caspase-8	Cell Signaling	1:500
7	Caspase-9	Cell Signaling	1:500
8	RARP-1	Cell Signaling	1:500
9	GRP78	Millipore	1:8,000
10	GRP94	Millipore	1:8,000
11	p-ASK	Santa Cruz Biotechnology	1:800
12	ASK	Santa Cruz Biotechnology	1:1,000
13	TRAF2	Cell Signaling	1:1,000
14	IRE1 α	Millipore	1:800
15	Caspase-4	Cell Signaling	1:500
16	Caspase-12	Cell Signaling	1:500
17	β -actin	Santa Cruz Biotechnology	1:800
18	p-JNK	Cell Signaling	1:500
19	Tubulin	Santa Cruz Biotechnology	1:800

by Tukey multiple comparison test was used when three or more groups were compared. In all cases, values of $P < 0.05$ were considered statistically significant.

Results

Effect of Wogonin on Cytotoxicity of SK-N-BE2 and IMR-32 Cells

We performed the MTT assay to evaluate the effect of wogonin against SK-N-BE2 and IMR-32 cells' cytotoxicity. According to the pre-experiment, we applied wogonin in the doses of 10, 25, 50, 75, 100, and 150 μ M. As illustrated in Fig. 1a, b, wogonin inhibited the cell viability of SK-N-BE2 and IMR-32 cells in a dose-dependent manner and the effect was predominant at 50–150 μ M. In addition, we applied 75 μ M wogonin in the time of 6, 12, 24, 48, 72, and 96 h. We found that 75 μ M wogonin inhibited the cell viability of SK-N-BE2 and IMR-32 cells in a time-dependent manner (Fig. 1c, d) and the effect was predominant at 48–96 h. Therefore, SK-N-BE2 and IMR-32 cells treated with 75 μ M wogonin for 48 h was chosen for the subsequent experiments.

Effect of Wogonin on Apoptosis of SK-N-BE2 and IMR-32 Cells

To further investigate whether wogonin could induce the apoptosis of SK-N-BE2 and IMR-32 cells, the cells were treated with 75 μ M wogonin for 48 h. As shown in Fig. 2a, b, Annexin V-FITC/PI staining analysis showed that the percentages of apoptotic cells were increased in SK-N-BE2 and IMR-32 cells treatment with 75 μ M wogonin for 48 h.

Effect of Wogonin on Activation of Caspases Family in SK-N-BE2 and IMR-32 Cells

The caspase pathway, which is activated both extrinsically and intrinsically, is the major mechanism of apoptosis in most cellular systems (Fadeel et al. 2005). Treatment of SK-N-BE2 and IMR-32 cells with 75 μ M wogonin for 48 h indeed resulted in strong activation of cleaved caspase-8, caspase-9, and caspase-3. The data in Fig. 3 clearly indicated that wogonin activated the intrinsic caspase cascade leading to caspase-9 followed by caspase-3 activation that resulted in poly ADP-ribose polymerase (PARP)-1 cleavage in SK-N-BE2 and IMR-32 cells.

Effect of Wogonin on Expressions of Bcl-2 Family Proteins in SK-N-BE2 and IMR-32 Cells

The proteins in Bcl-2 family are major regulatory proteins associated with apoptosis. As depicted in Fig. 4, Bcl-2, an

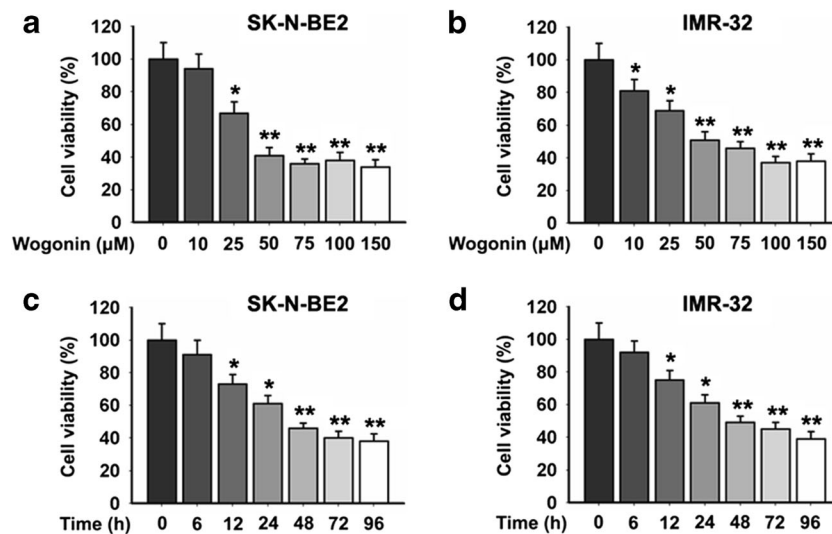


Fig. 1 Effect of wogonin on cell viability in SK-N-BE2 and IMR-32 cells. Cell viability was assayed by the MTT method. **a** Effect of different doses of wogonin on the cell viability of SK-N-BE2 cells. **b** Effect of different doses of wogonin on the cell viability of IMR-32 cells. **c** Effect of 75 μM wogonin on the cell viability of SK-N-BE2

cells in a time-dependent manner. **d** Effect of 75 μM wogonin on the cell viability of IMR-32 cells in a time-dependent manner. All data are presented as means±SD. (*n*=6, **P*<0.05, ***P*<0.01 significantly different from the control group). The control group set at 100 %

apoptotic suppressor, was markedly lower after treatment with 75 μM wogonin for 48 h. In contrast, Bax and Bid, the pro-apoptotic proteins, expressions were significantly higher after treatment with 75 μM wogonin for 48 h, suggesting a balance towards induction of mitochondrial dysfunction.

Effect of Wogonin on Release of Cytochrome c in SK-N-BE2 and IMR-32 Cells

Cytochrome c is released from the mitochondria into the cytosol to activate mitochondria-dependent caspase cascade.

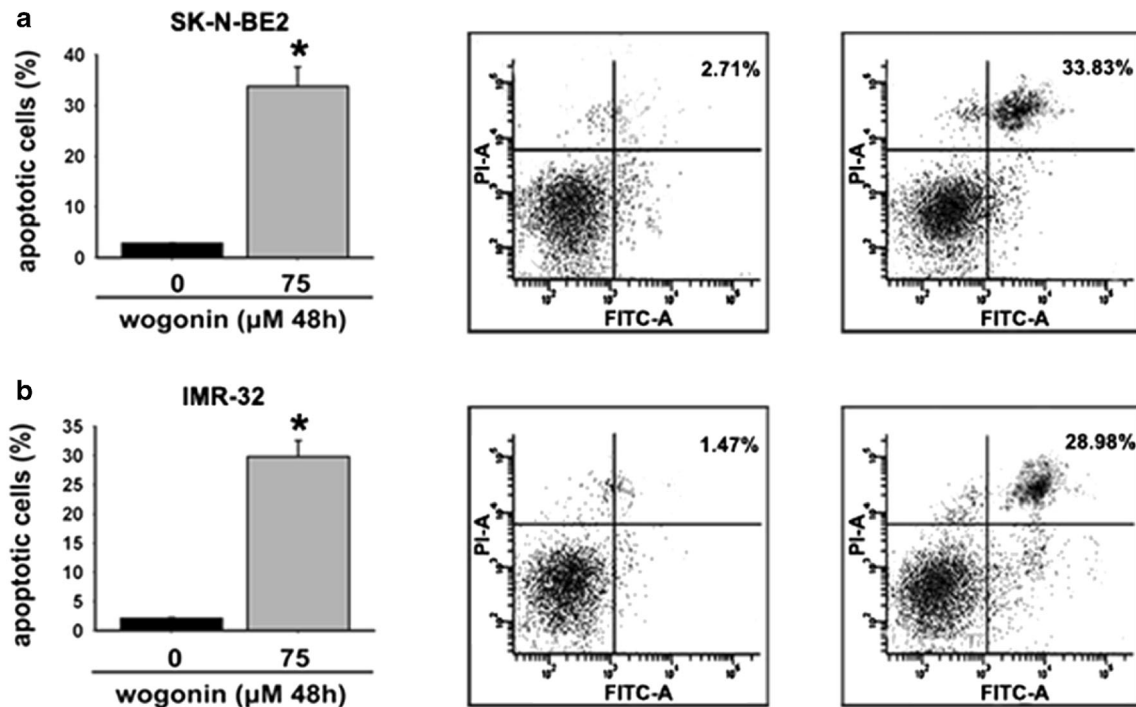
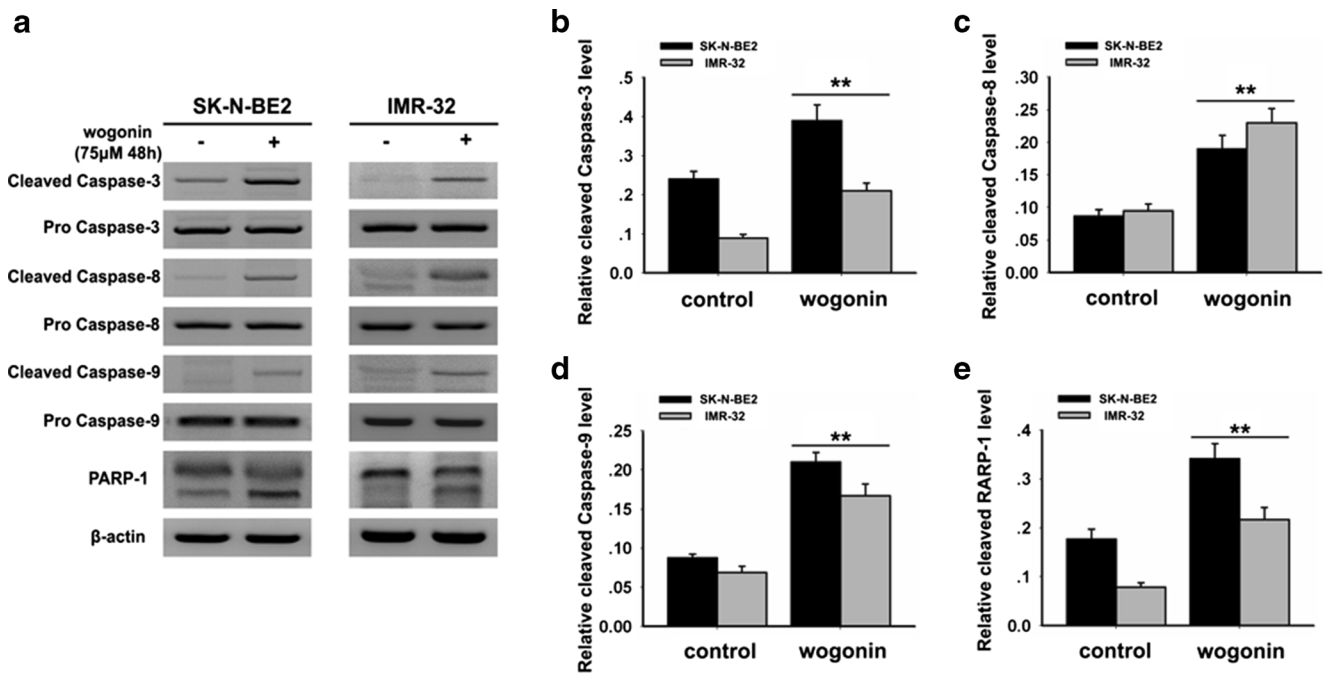


Fig. 2 Effect of wogonin on the apoptosis rate in SK-N-BE2 and IMR-32 cells. Leukemia cell apoptosis discriminated by the Annexin V/PI flow cytometric assay method, which could detect cells in an earlier stage of the apoptotic pathway and distinguish among apoptotic cells. **a** Wogonin

treatment (75 μM) induced apoptosis of SK-N-BE2 cells. **b** Wogonin treatment (75 μM) induced apoptosis of IMR-32 cells. Data were shown as mean±SEM (*n*=6, **P*<0.05 significantly different from the control group)

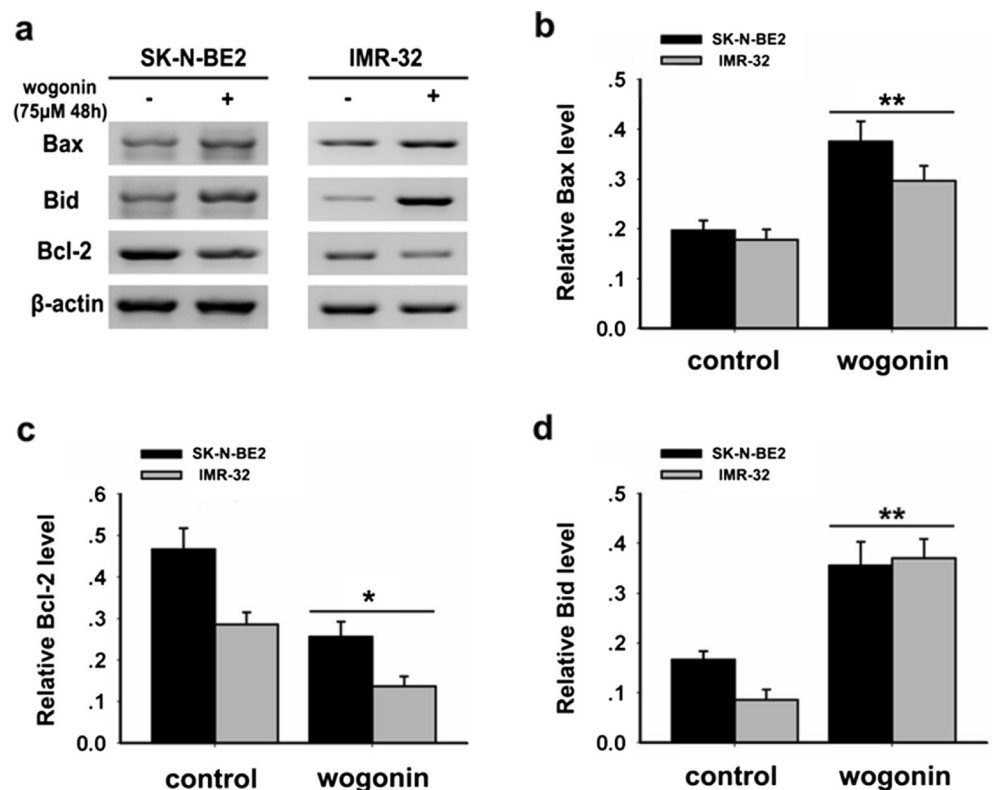


Next, we examined the levels of cytochrome c in both mitochondrial and cytosolic fractions. Treatment of SK-N-BE2

and IMR-32 cells with 75 μM wogonin for 48 h decreased the level of cytochrome c in the mitochondria and

and IMR-32 cells with 75 μM wogonin for 48 h decreased the level of cytochrome c in the mitochondria and

Fig. 4 Effect of wogonin on the activation of mitochondrial-dependent apoptotic signaling in SK-N-BE2 and IMR-32 cells. **a** The expressions of Bax, Bid, and Bcl-2 were measured in SK-N-BE2 and IMR-32 cells with 75 μM wogonin treatment for 48 h by western blotting assay. **b–d** The bar chart showed the ratio of Bax, Bid, and Bcl-2 to β-actin at each group. These data are means±SEM. ($n=6$, $*P<0.05$, $**P<0.01$ significantly different from the control group)



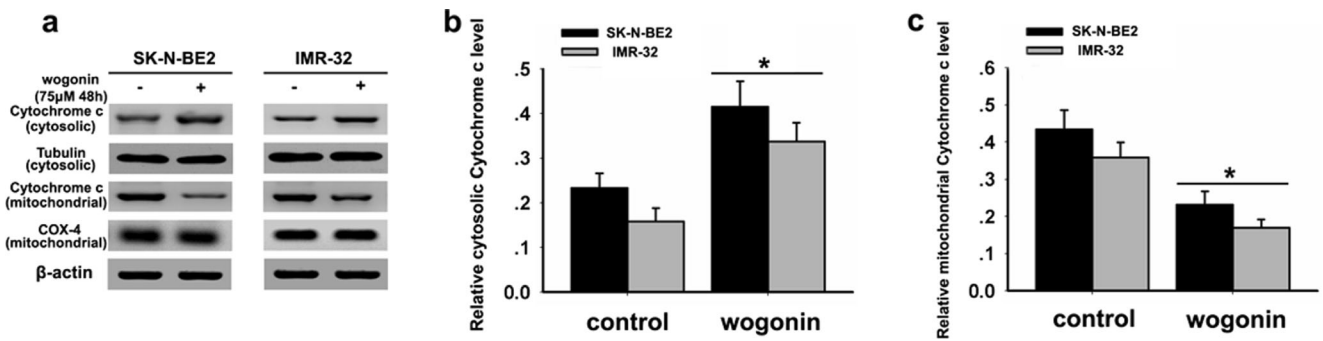


Fig. 5 Effect of wogonin on the release of cytochrome c in SK-N-BE2 and IMR-32 cells. **a** The levels of cytochrome c in both mitochondrial and cytosolic fractions was measured in SK-N-BE2 and IMR-32 cells with 75 μM wogonin treatment for 48 h by western blotting assay. Expression of COX-4 and tubulin was used for monitoring mitochondrial release of

cytochrome c into the cytosol. **b, c** The bar chart showed the ratio of cytochrome c to COX-4 in mitochondrial and tubulin in cytosolic fractions at each group. These data are means±SEM. (*n*=6, **P*<0.05 significantly different from the control group)

concomitantly increased the level of cytochrome c in the cytosol, confirming the mitochondrial release of cytochrome c into the cytosol (Fig. 5).

also induced activation of caspase-4 and caspase-12 (Fig. 6a–e), which were closely related to ER stress-induced cell death. Collectively, these data suggested a role for ER stress in wogonin-induced apoptosis of SK-N-BE2 and IMR-32 cells.

Effect of Wogonin on ER Stress-Associated Proteins of SK-N-BE2 and IMR-32 Cells

Because ER stress was one of the mechanisms of apoptotic process, we examined the effect of wogonin on the expressions of ER stress-associated proteins in SK-N-BE2 and IMR-32 cells. Firstly, using western blot analysis, we examined whether wogonin affected expression of ER stress-associated proteins, such as glucose-regulated protein (GRP78)/the immunoglobulin heavy chain binding protein (Bip) and GRP94/gp96. Results showed expressions of GRP78 and GRP94 increased after treatment with 75 μM wogonin for 48 h (Fig. 6a–c). Wogonin

Wogonin Induces Mitochondrial Dysfunction Through IRE1α-Dependent Pathway in SK-N-BE2 and IMR-32 Cells

Previous studies have shown that the c-Jun N-terminal kinase (JNK) pathway could be activated by ER stress following recruitment of TRAF2 by the IRE1 cytosolic kinase to form a TRAF2-ASK1-IRE1 complex (Urano et al. 2000). As depicted in Fig. 7, increased expression of IRE1α, TRAF2, p-ASK, and p-JNK was markedly measured after treatment with 75 μM wogonin for 48 h. To further explore the role of

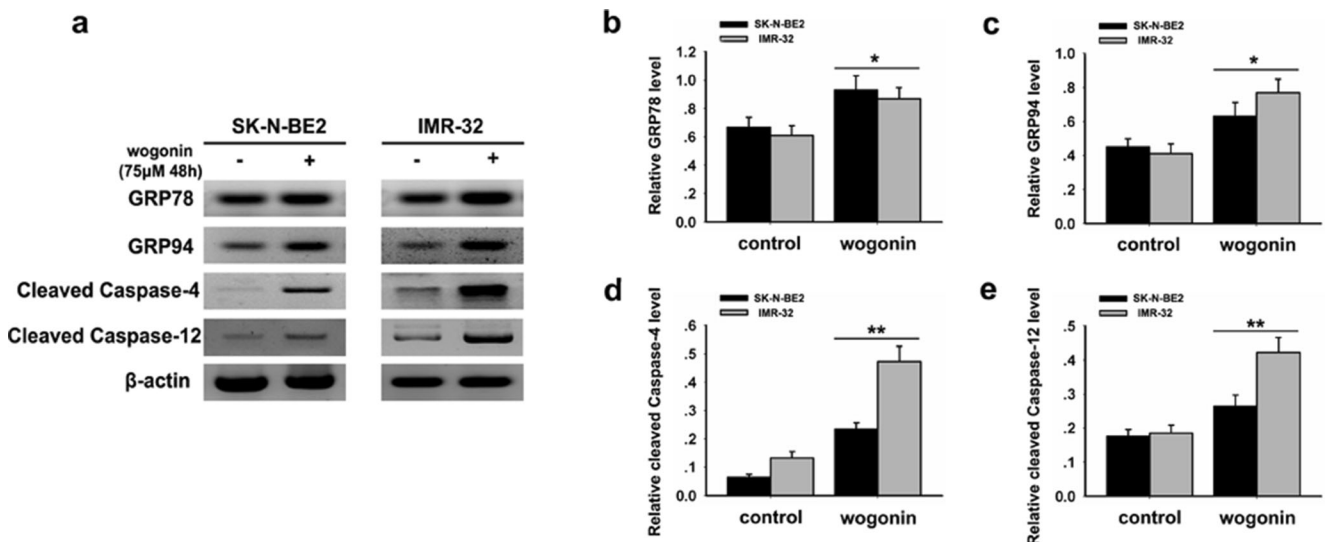
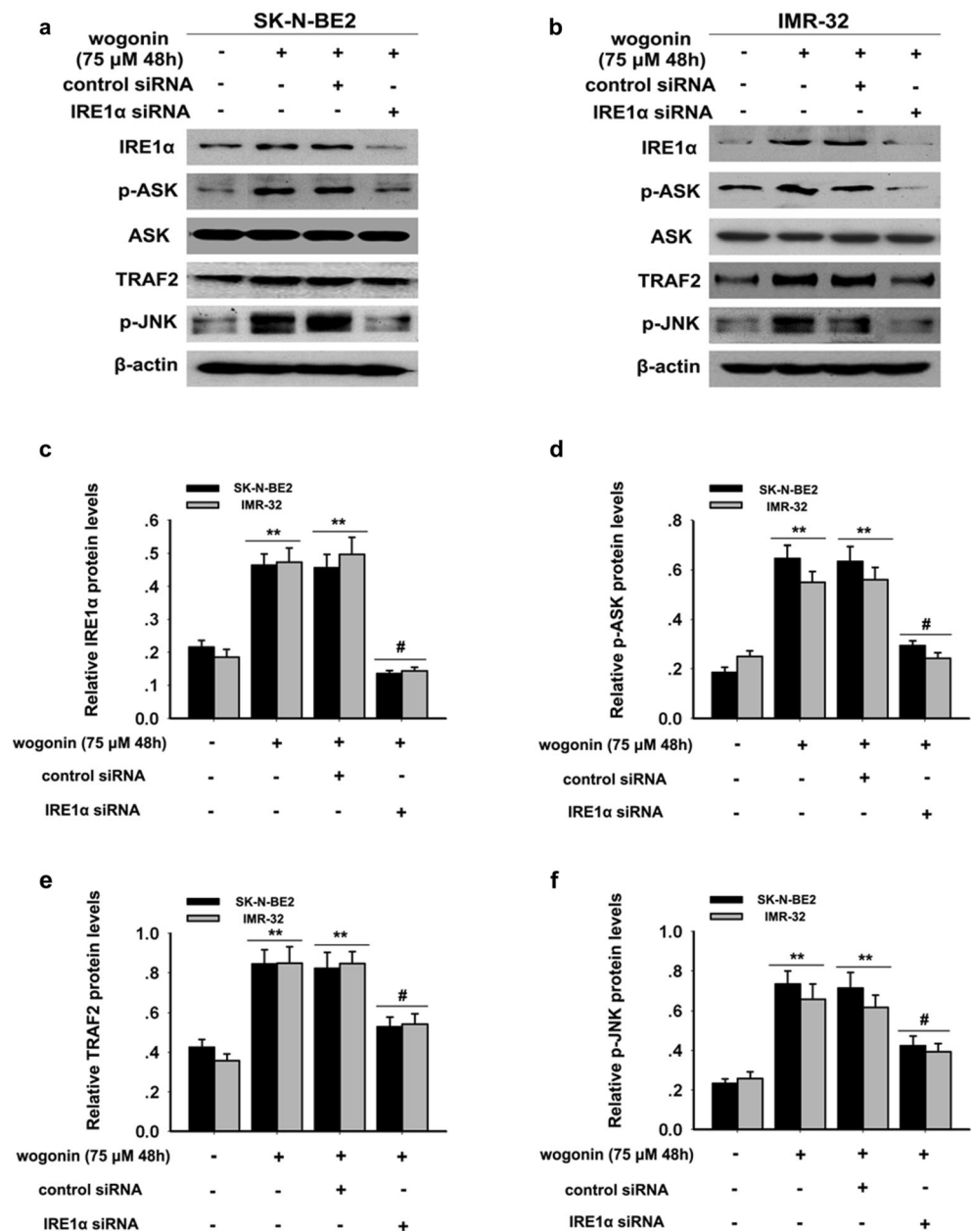


Fig. 6 Effect of wogonin on the ER stress in SK-N-BE2 and IMR-32 cells. **a** The expressions of GRP78, GRP94, cleaved caspase-4, and caspase-12 were measured in SK-N-BE2 and IMR-32 cells with 75 μM wogonin treatment for 48 h by western blot analysis. **b–e** The bar chart

showed the ratio of these ER stress-related proteins to β-actin at each group. These data are means±SEM. (*n*=6, **P*<0.05, ***P*<0.01 significantly different from the control group)

Fig. 7 Effect of wogonin on the IRE1 α -dependent pathway in SK-N-BE2 and IMR-32 cells. **a, b** The expression levels of IRE1 α , TRAF2, p-ASK1, and p-JNK were measured in SK-N-BE2 and IMR-32 cells with 75 μ M wogonin by western blot analysis. SK-N-BE2 and IMR-32 cells were transfected with either control siRNA or with a specific IRE1 α siRNA sequence at 50 nM for 24 h. **c, f** The bar chart showed the ratio of IRE1 α , TRAF2, p-ASK1, and p-JNK to β -actin at each group. These data are means \pm SEM. ($n=6$, $*P<0.05$, $**P<0.01$ significantly different from the control group; $\#P<0.05$ significantly different from the wogonin treatment group)



IRE1 α -dependent pathway in wogonin-induced apoptosis, SK-N-BE2 and IMR-32 cells were transfected with or without IRE1 α siRNA for 24 h. Twenty-four hours later, SK-N-BE2 and IMR-32 cells were treated with wogonin for 48 h, and levels of IRE1 α , p-ASK1, p-JNK, and TRAF2 were analyzed by western blot. As shown in Fig. 7, the activation of IRE1 α -dependent pathway was markedly inhibited in cells transfected with IRE1 α siRNA following treatment with wogonin. Furthermore, inhibition of IRE1 α markedly inhibited wogonin-induced cytotoxic effects (Fig. 8a), increase of apoptotic ratio (Fig. 8b), and cleaved caspase-3 and caspase-9 (Fig. 9a–d) and Bax/Bcl-2 (Fig. 9a–f) in both cell lines. These results indicated that the proapoptotic effects of wogonin in SK-N-BE2 and IMR-32

cells were associated with the activation of the IRE1 α -dependent pathway.

Discussion

Recently, traditional Chinese medicines have been followed with interest as a new source of anti-cancer drugs (Li-Weber 2009). So far, much effort has been put on the development of agents that can effectively induce the apoptosis of cancer cells. Clinically, *S. baicalensis* has been used in Chinese medicine as a traditional adjuvant for cancer chemotherapy. However, the molecular mechanisms of their actions are still largely

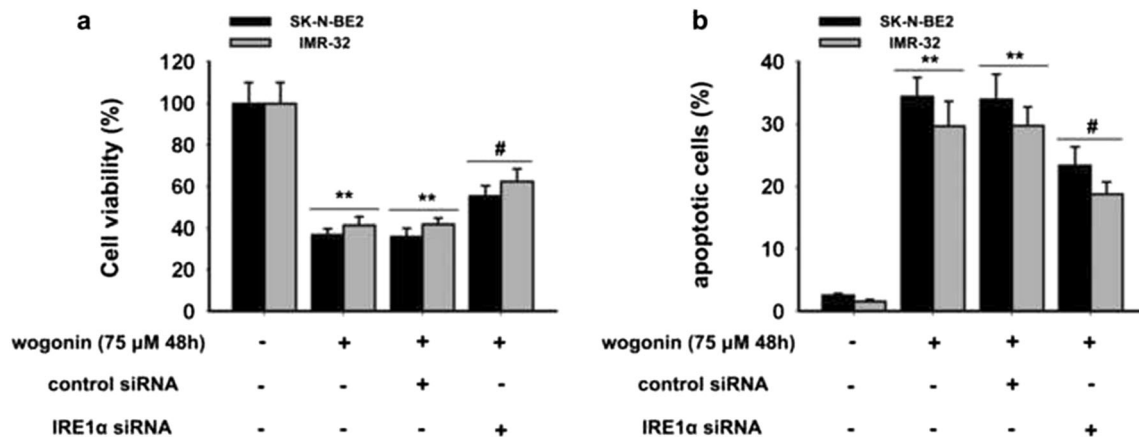


Fig. 8 Effect of IRE1α on wogonin-induced cytotoxic effects and apoptosis in SK-N-BE2 and IMR-32 cells. **a** Effect of wogonin on the cell viability of SK-N-BE2 and IMR-32 cells after transfected with either control siRNA or with a specific IRE1α siRNA sequence at 50 nM for 24 h. **b** Effect of wogonin on the level of apoptosis in SK-N-BE2 and

IMR-32 cells after transfected with either control siRNA or with a specific IRE1α siRNA sequence at 50 nM for 24 h. These data are means±SEM. (n=6, *P<0.05, **P<0.01 significantly different from the control group; #P<0.05 significantly different from the wogonin treatment group)

unknown. This study presents data showing that wogonin, a major constituent of *S. baicalensis*, induces apoptosis of human malignant neuroblastoma cells by up-regulating mitochondrial dysfunction and ER stress-dependent pathway. These results implied that an adjuvant therapy with wogonin might have potential therapeutic benefits for malignant neuroblastoma.

In this study, we firstly investigated whether wogonin induced apoptosis in SK-N-BE2 and IMR-32 cells. MTT assay demonstrated that wogonin inhibited cell viability of SK-N-BE2 and IMR-32 cells in a dose- and time-dependent manner, and the effect was predominant with a concentration of 75 μM wogonin for 48 h. Meanwhile, flow cytometry analysis indicated that 75 μM wogonin for 48 h effectively induced N-BE2 and IMR-32 cells apoptosis. These results suggested that wogonin-induced inhibition of cell viability of SK-N-BE2 is a causal factor responsible for their own apoptosis.

Apoptosis is an active gene-directed form of cell death that is different from cell necrosis with respect to its morphological, biochemical, pharmacological, and biological significance (Wang et al. 2014). Apoptosis is a widely accepted important mechanism that contributes to cell growth reduction and is a major method of anti-cancer properties to eliminate cancer cells (Kelloff et al. 2000). Apoptosis is controlled by both extrinsic and intrinsic pathways (Chiantore et al. 2009). The extrinsic pathway involves the death receptor, in which the death domains target caspase-8 combined with their corresponding ligands. The activation of caspase-8 then activates caspase-3 to ultimately induce apoptosis. Caspase-3 is a member of the caspase family enzymes, which are the major inducers of apoptosis. Caspase-3 activity is often measured in the context of research into anti-tumor drugs that target apoptosis (Du et al. 2013; He et al. 2014). In the present study, we investigated whether wogonin could induce apoptosis of SK-N-BE2 and IMR-32 cells through regulating the extrinsic

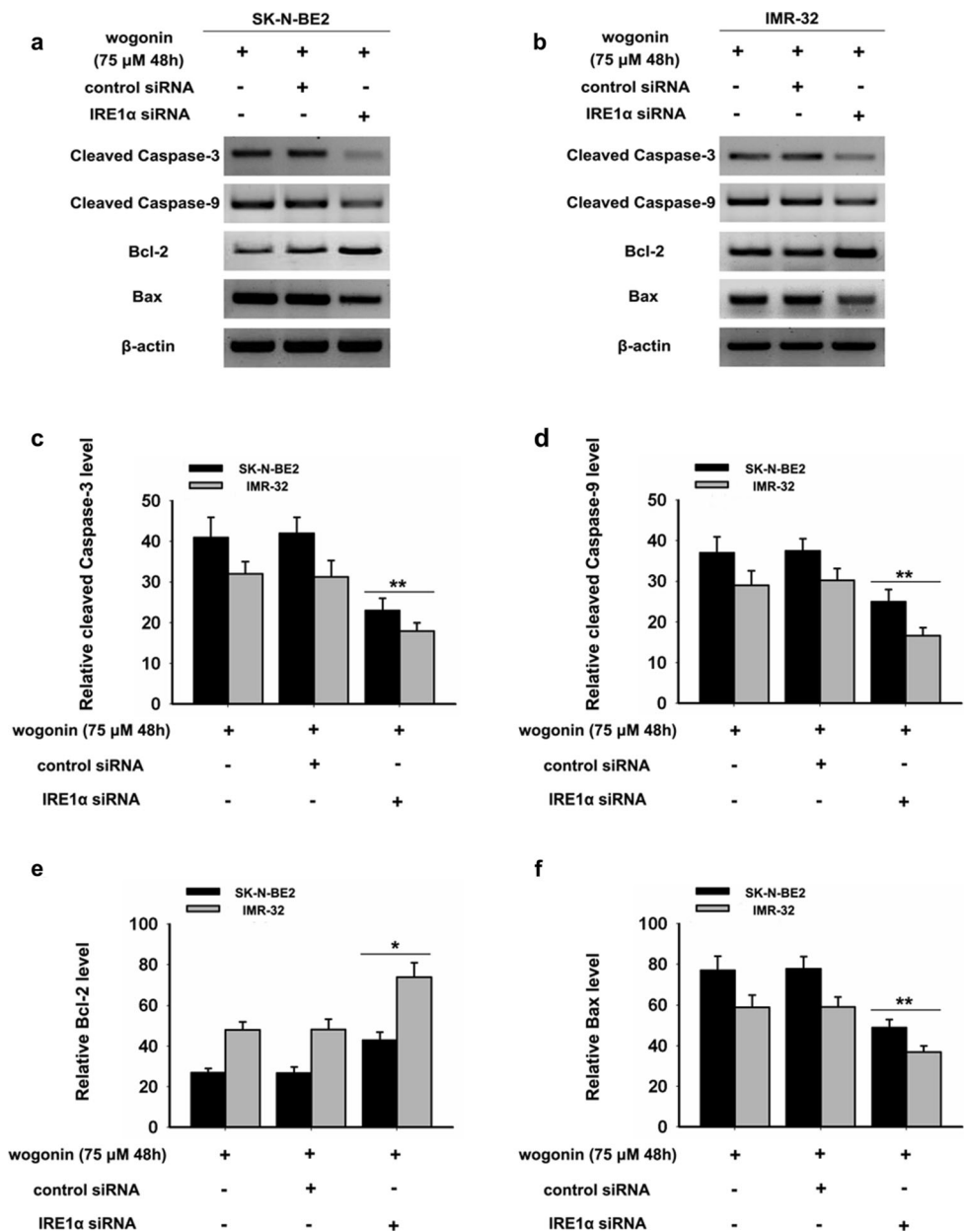
pathway. The expressions of cleaved caspase-3, caspase-8, and caspase-9 were increased after being treated with 75 μM wogonin for 48 h, which might be the reason for pro-apoptotic effects of wogonin in SK-N-BE2 and IMR-32 cells.

The intrinsic pathway (mitochondrial pathway) of apoptosis is associated with DNA damage. DNA-damaging reagents directly or indirectly activate the mitochondrial pathway resulting in the release of mitochondrial cytochrome c into the cytoplasm. Cytochrome c combines with the caspase-9 precursor to form an apoptosis complex. The activation of caspase-9 then activates caspase-3 and PARP to induce apoptosis (Meier and Vousden 2007). We examined the levels of cytochrome c in both mitochondrial and cytosolic fractions. Treatment of SK-N-BE2 and IMR-32 cells with 75 μM wogonin for 48 h caused the mitochondrial release of cytochrome c into the cytosol leading to the activation of the final executioner caspase-3 that fragmented the DNA repair enzyme PARP-1, fulfilling a pre-requisite of DNA fragmentation for apoptotic death in both malignant neuroblastoma cell lines.

Bcl-2 family members are key regulators of apoptosis. The ratio between anti-apoptotic (Bcl-2) and pro-apoptotic (Bax and Bid) members in Bcl-2 family is considered to be a determinant factor for tissue homeostasis because it influences the sensitivity of cells to inducers of apoptosis (Brenner and Mak 2009; Youle and Strasser 2008). Our result showed a decrease of Bcl-2 protein following wogonin treatment, while increased expressions of pro-apoptotic Bax and Bid were observed in wogonin-treated SK-N-BE2 and IMR-32 cells. Therefore, wogonin might initiate mitochondrial dysfunction which induced caspase-dependent apoptotic signaling.

Nowadays, there has been increasing awareness regarding the role of the ER stress in the homeostasis of the cancer cell (Lin et al. 2011; Tsai et al. 2012; Xu et al. 2013; Yang et al. 2012). ER stress occurs when ER homeostasis is lost due to an

Fig. 9 Effect of IRE1 α on wogonin-induced mitochondrial-dependent apoptotic signaling in SK-N-BE2 and IMR-32 cells. **a, b** The expression of cleaved caspase-3, cleaved caspase-9, Bax, and Bcl-2 were measured in SK-N-BE2 and IMR-32 cells with 75 μ M wogonin treatment for 48 h by western blotting assay. **c–f** The bar chart showed the ratio of cleaved caspase-3, cleaved caspase-9, Bax, and Bcl-2 to β -actin at each group. These data are means \pm SEM. ($n=6$, * $P<0.05$, ** $P<0.01$ significantly different from the wogonin treatment group)



overload of protein folding in the ER. Abnormal ER function can cause ER stress, which results in unfolded protein response, including the key signaling proteins: IRE1-TRAF2-ASK, act as the sensors of ER stresses. Although the UPR is primarily a pro-survival response, under the circumstance of prolonged or enhanced ER stress, the UPR switches to cause cell apoptosis (Boyce and Yuan 2006; Breckenridge et al. 2003; Brenner and Mak 2009). Here, we observed that wogonin up-regulated the expressions of p-ASK, IRE1 α , and TRAF2 in SK-N-BE2 and IMR-32 cells. Activation of caspase-4 and caspase-12 localized in the ER, exerting the pro-apoptotic actions of ER stress, has been reported (Boyce and Yuan 2006; Breckenridge et al. 2003). In agreement with the effect of wogonin on ER stress, wogonin induced

expression and activation of caspase-4 and caspase-12. Some ER chaperone genes, including GRP78 and GRP94, were activated after 75 μ M wogonin treatment for 48 h in SK-N-BE2 and IMR-32 cells. This finding suggested that the apoptosis induced by wogonin in SK-N-BE2 and IMR-32 cells is with ER stress involvement.

Previous studies have also shown that ER stress may activate the mitogen-activated protein kinases. The JNK pathway was activated following the binding of IRE1 to the scaffold molecule TRAF2 (Urano et al. 2000; Nishitoh et al. 2002) and consequently activated ASK1/JNK (Nishitoh et al. 2002). In the present study, the activation of ASK1 and IRE1 α was consistent with the activation of the JNK pathway suggesting that JNK was activated in response to wogonin-induced ER

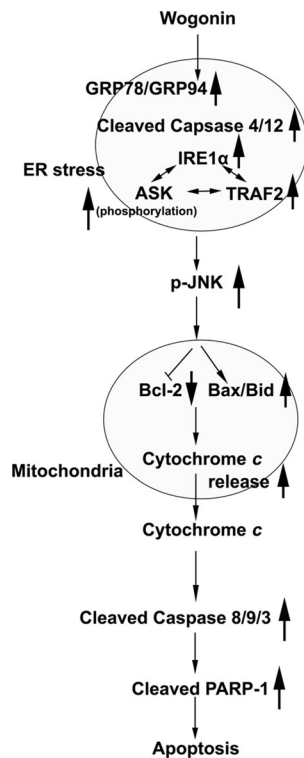


Fig. 10 Possible mechanisms by which wogonin-induced apoptosis in SK-N-BE2 and IMR-32 cells. ER stress are activated by up-regulation of GRP78, GRP94, activation of caspase-4, caspase-12, p-JNK IRE1 α , TRAF2, and p-ASK1 after wogonin treatment, in order to increase the expression in Bax, Bid, Cyt c release, caspase-3, caspase-8, caspase-9 and PARP-1 activity, and subsequent apoptosis

stress. The almost complete loss of JNK activation and wogonin-induced apoptosis by inhibition of IRE1 α strongly argues that IRE1 α -dependent pathway is an essential cell death-signaling pathway in response to wogonin treatment.

In summary, this study demonstrated that wogonin induced mitochondrial dysfunction, ER stress, and caspase-dependent apoptosis in human malignant neuroblastoma SK-N-BE2 and IMR-32 cells lines, which was associated with the up-regulated IRE1 α -dependent pathway (Fig. 10). Therefore, we suggested that wogonin might be effective in the treatment of malignant neuroblastoma.

Conflict of Interest The authors declare no conflict of interest.

References

- Boyce M, Yuan J (2006) Cellular response to endoplasmic reticulum stress: a matter of life or death. *Cell Death Differ* 13:363–373
- Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC (2003) Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene* 22:8608–8618
- Brenner D, Mak TW (2009) Mitochondrial cell death effectors. *Curr Opin Cell Biol* 21:871–877
- Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 3:203–216
- Chakrabarti M, Banik NL, Ray SK (2013) Sequential hTERT knockdown and apigenin treatment inhibited invasion and proliferation and induced apoptosis in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells. *J Mol Neurosci* : MN 51:187–198
- Chiantore MV, Vannucchi S, Mangino G, Percario ZA, Affabris E, Fiorucci G, Romeo G (2009) Senescence and cell death pathways and their role in cancer therapeutic outcome. *Curr Med Chem* 16: 287–300
- Dong P, Zhang Y, Gu J, Wu W, Li M, Yang J, Zhang L, Lu J, Mu J, Chen L, Li S, Wang J, Liu Y (2011) Wogonin, an active ingredient of Chinese herb medicine *Scutellaria baicalensis*, inhibits the mobility and invasion of human gallbladder carcinoma GBC-SD cells by inducing the expression of maspin. *J Ethnopharmacol* 137:1373–1380
- Du LQ, Wang Y, Xu C, Cao J, Wang Q, Zhao H, Fan FY, Wang B, Katsube T, Fan SJ, Liu Q (2013) Radiation-sensitising effects of antennapedia proteins (ANTP)-SmacN7 on tumour cells. *Int J Mol Sci* 14:24087–24096
- Fadeel B, Orrenius S (2005) Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med* 258: 479–517
- Gao J, Morgan WA, Sanchez-Medina A, Corcoran O (2011) The ethanol extract of *Scutellaria baicalensis* and the active compounds induce cell cycle arrest and apoptosis including upregulation of p53 and Bax in human lung cancer cells. *Toxicol Appl Pharmacol* 254: 221–228
- Gasiorowski K, Lamer-Zarawska E, Leszek J, Parvathaneni K, Yendluri BB, Blach-Olszewska Z, Aliev G (2011) Flavones from root of *Scutellaria baicalensis* Georgi: drugs of the future in neurodegeneration? *CNS Neurol Disord Drug Targets* 10:184–191
- He L, Lu N, Dai Q, Zhao Y, Zhao L, Wang H, Li Z, You Q, Guo Q (2013) Wogonin induced G1 cell cycle arrest by regulating Wnt/beta-catenin signaling pathway and inactivating CDK8 in human colorectal cancer carcinoma cells. *Toxicology* 312:36–47
- He K, Si P, Wang H, Tahir U, Chen K, Xiao J, Duan X, Huang R, Xiang G (2014) Crocetin induces apoptosis of BGC-823 human gastric cancer cells. *Mol Med Rep* 9:521–526
- Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 13:89–102
- Huang KF, Zhang GD, Huang YQ, Diao Y (2012) Wogonin induces apoptosis and down-regulates survivin in human breast cancer MCF-7 cells by modulating PI3K-AKT pathway. *Int Immunopharmacol* 12:334–341
- Huang Y, Xu Y, Cheng Q, Yu S, Gao Y, Shu Q, Yang C, Sun Y, Wang J, Xu F, Liang X (2014) The expression changes of myelin and lymphocyte protein (MAL) following optic nerve crush in adult rats retinal ganglion cells. *J Mol Neurosci* 54(4):614–621
- Irshad S, Pedley RB, Anderson J, Latchman DS, Budhram-Mahadeo V (2004) The Brn-3b transcription factor regulates the growth, behavior, and invasiveness of human neuroblastoma cells in vitro and in vivo. *J Biol Chem* 279:21617–21627
- Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW, Kopelovich L, Hawk ET, Lieberman R, Lawrence JA, Ali I, Viner JL, Sigman CC (2000) Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* 130:467S–471S
- Lee DH, Lee TH, Jung CH, Kim YH (2012) Wogonin induces apoptosis by activating the AMPK and p53 signaling pathways in human glioblastoma cells. *Cell Signal* 24:2216–2225
- Lin CC, Kuo CL, Lee MH, Lai KC, Lin JP, Yang JS, Yu CS, Lu CC, Chiang JH, Chueh FS, Chung JG (2011) Wogonin triggers apoptosis in human osteosarcoma U-2 OS cells through the endoplasmic

- reticulum stress, mitochondrial dysfunction and caspase-3-dependent signaling pathways. *Int J Oncol* 39:217–224
- Li-Weber M (2009) New therapeutic aspects of flavones: the anticancer properties of *Scutellaria* and its main active constituents wogonin, baicalein and baicalin. *Cancer Treat Rev* 35:57–68
- Meier P, Vousden KH (2007) Lucifer's labyrinth—ten years of path finding in cell death. *Mol Cell* 28:746–754
- Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 16:1345–1355
- Schor NF (2009) New approaches to pharmacotherapy of tumors of the nervous system during childhood and adolescence. *Pharmacol Ther* 122:44–55
- Tsai CF, Yeh WL, Huang SM, Tan TW, Lu DY (2012) Wogonin induces reactive oxygen species production and cell apoptosis in human glioma cancer cells. *Int J Mol Sci* 13:9877–9892
- Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664–666
- Wang Y, Wan C, Yu S, Yang L, Li B, Lu T, Bi Y, Jiang J, Cui G (2014) Upregulated expression of NF- κ B contributes to neuronal apoptosis via proapoptotic protein bim in rats' brain hippocampus following middle cerebral artery occlusion (MCAO). *J Mol Neurosci* 52:552–565
- Xu M, Lu N, Zhang H, Dai Q, Wei L, Li Z, You Q, Guo Q (2013) Wogonin induced cytotoxicity in human hepatocellular carcinoma cells by activation of unfolded protein response and inactivation of AKT. *Hepatol Res* 43:890–905
- Xu Y, Yang L, Yu S, Shu Q, Yang C, Wang J, Xu F, Sang A, Liang X (2014) Spatiotemporal changes in NFATc4 expression of retinal ganglion cells after light-induced damage. *J Mol Neurosci* 53:69–77
- Yang Y, Li XJ, Chen Z, Zhu XX, Wang J, Zhang LB, Qiang L, Ma YJ, Li ZY, Guo QL, You QD (2012) Wogonin induced calreticulin/annexin A1 exposure dictates the immunogenicity of cancer cells in a PERK/AKT dependent manner. *PLoS One* 7:e50811
- Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 9:47–59
- Zhang M, Liu LP, Chen Y, Tian XY, Qin J, Wang D, Li Z, Mo SL (2013) Wogonin induces apoptosis in RPMI 8226, a human myeloma cell line, by downregulating phospho-Akt and overexpressing Bax. *Life Sci* 92:55–62