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Protective Effects of Fisetin Against 6-OHDA-Induced Apoptosis by Activation of PI3K-Akt Signaling in Human Neuroblastoma SH-SY5Y Cells

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Abstract

6-Hydroxydopamine (6-OHDA) induces the production of reactive oxygen species (ROS) that are associated with various neurodegenerative diseases such as Parkinson's disease. 3,3′,4′,7-Tetrahydroxyflavone (fisetin), a plant flavonoid has a variety of physiological effects such as antioxidant activity. In this study, we investigated the molecular mechanism of the neuroprotective effects of fisetin against 6-OHDA-induced cell death in human neuroblastoma SH-SY5Y cells. 6-OHDA-mediated cell toxicity was reduced in a fisetin concentration-dependent manner. 6-OHDA-mediated elevation of the expression of the oxidative stress-related genes such as hemeoxygenase-1, NAD(P)H dehydrogenase quinone 1, NF-E2-related factor 2, and γ-glutamate-cysteine ligase modifier was suppressed by fisetin. Fisetin also lowered the ratio of the proapoptotic Bax protein and the antiapoptotic Bcl-2 protein in SH-SY5Y cells. Moreover, fisetin effectively suppressed 6-OHDA-mediated activation of caspase-3 and caspase-9, which leads to the cell death, while, 6-OHDA-induced caspase-3/7 activity was lowered. Furthermore, fisetin activated the PI3K-Akt signaling, which inhibits the caspase cascade, and fisetin-mediated inhibition of 6-OHDA-induced cell death was negated by the co-treatment with an Akt inhibitor. These results indicate that fisetin protects 6-OHDA-induced cell death by activating PI3K-Akt signaling in human neuronal SH-SY5Y cells. This is the first report that the PI3K-Akt signaling is involved in the fisetin-protected ROS-mediated neuronal cell death.

Keywords Fisetin · 6-OHDA · Caspase · PI3K-Akt · SH-SY5Y cells

Abbreviations

PD	Parkinson's disease
ROS	Reactive oxygen species
6-OHDA	6-Hydroxydopamine
fisetin	3,3′,4′,7-Tetrahydroxyflavone
ERK	Extracellular signal-regulated kinase
HRP	Horseradish peroxidase
DCDFA	2′,7′-Dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
LDH	Lactate dehydrogenase
qPCR	Quantitative PCR
HO-1	Hemeoxygenase-1
NQO1	NAD(P)H dehydrogenase quinone 1
NRF2	NF-E2-related factor 2
GCLM	γ-glutamate-cysteine ligase modifier
PI3K	Phosphoinositide 3-kinase
Akti	Akt inhibitor

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Introduction

Parkinson's disease (PD) is a neurodegenerative disease that results from the loss of dopaminergic neurons in the substantia nigra [1]. Approximately 1–2% of the population aged above 65 years is affected by PD [2, 3]. An imbalance exists between the production and elimination of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide in PD, which induces the dysfunction or death of neuronal cells [1, 4]. Although its etiology is still unclear, PD is associated with mitochondrial dysfunction, oxidative stress, and activation of the caspase cascade in apoptosis [1].

6-Hydroxydopamine (6-OHDA) is commonly used as a neurotoxin in the study of PD, because it has a similar molecular structure to dopamine [5], and generates intracellular ROS [6, 7]. Neuronal cell death by oxidative stress has been associated with the activation of apoptotic cascades [8]. Thus, oxidative stress may be responsible for dopaminergic neurodegeneration by activating the apoptotic cascades. In fact, antioxidants prevent the progression of PD [9]. Therefore, inhibiting the generation of intracellular ROS may be useful for the prevention and treatment of PD.

Understanding of molecular mechanisms underlying ROS-induced neurodegenerative disease could allow the development of a strategy to prevent and treat PD. Various therapeutics for the treatment of PD have been developed [10]. Moreover, many researchers have reported different strategies to lower oxidative stress in dopaminergic neurons. Some natural products have the ability to prevent and treat neurodegenerative diseases. Antioxidants such as vitamin C, vitamin E, and glutathione in fruits and vegetables repress oxidative stress-induced neuronal cell death [11, 12]. Flavonoids are naturally-occurring polyphenolic compounds that are widely present in a variety of fruits and vegetables [13]. They exert a range of biological and physiological effects; e.g., anticancer, antioxidative, anti-inflammatory, and antiobesity [14–18]. Thus, the antioxidant properties of natural products are now of interest with respect to potential application in the treatment of PD. In fact, neuroprotective properties of a plant flavonoid, 3,3′,4′,7-tetrahydroxyflavone (fisetin; Fig. 1) that we investigated in this study have been reported in vitro [19–21] and in vivo [22–25]. Thus, fisetin plays important roles in the protection against ROS-mediated cell damage in neuronal cells. It has been reported that fisetin activates the extracellular signal-regulated kinase (ERK) signaling in neuronal cells [19, 22]. Although many studies about the biological effects of fisetin in neuronal cells have been reported, the protective mechanism of neuronal cell death by fisetin has not been well elucidated.

In this study, we found that fisetin protects the cell death though inhibition of the 6-OHDA-activated caspase cascade via enhancement of the PI3K-Akt signaling in human neuronal SH-SY5Y cells. This is the first report that the PI3K-Akt signaling is involved in the protection of ROS-mediated cell death by fisetin in neuronal cells.

Experimental Procedures

Materials

Fisetin was obtained from Sigma (St. Louis, MO, USA). 6-OHDA was purchased from WAKO Pure Chemicals (Osaka, Japan). Anti-Akt, anti-phospho-Akt, anti-Bax and anti-Bcl-2 polyclonal antibodies were from Santa Cruz Biotech. (Santa Cruz, CA, USA), and anti-cleaved caspase-3 and anti-cleaved caspase-9 polyclonal antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin monoclonal antibody was from Sigma. Secondary antibodies such as anti-rabbit and anti-mouse IgG antibodies conjugated with horseradish peroxidase (HRP) were from Santa Cruz Biotech. 2′,7′-Dichlorofluorescein diacetate (DCFDA) and Akt inhibitor X were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

Cell Culture

Human neuronal SH-SY5Y cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10%(v/v) fetal bovine serum and antibiotics (Penicillin, 100 U/mL and streptomycin 10 mg/mL; Nacalai Tesque, Kyoto, Japan). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

Cell Viability Test and Lactate Dehydrogenase (LDH) Release Assay

SH-SY5Y cells were seeded in a 96-well plate at a density of 5×10^4 cells per well. The cells were cultured in DMEM with fisetin (0–100 μM) for 24 h. Cell toxicity was measured by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocols.

LDH release into the medium was measured with a Cytotoxicity LDH Assay Kit-WST (Dojindo) according to the instructions provided by the manufacturer.

Intracellular ROS Level

SH-SY5Y cells were pretreated in DMEM with fisetin (10 μM) for 3 h, and they were cultured in DMEM containing 6-OHDA (50 μM) alone for 24 h. Subsequently, 20 μM DCFDA was added to each well, and the cells were further incubated at 37 °C for 30 min, followed by washing with PBS. Fluorescence was observed using a fluorescence microscope (CKX-41-FL, Olympus, Tokyo, Japan). Cells
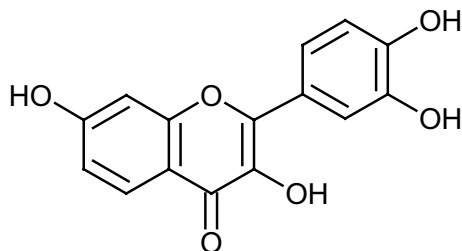

Fig. 1 Structure of fisetin

were then solubilized with PBS containing 0.1% (v/v) Triton X-100, sonicated, and centrifuged (20,000×g, 10 min at 4 °C) to remove the cell debris. The supernatant was transferred to 96-well black plates to measure the fluorescence at 488 nm excitation and 523 nm emission wavelengths using the EnSpire 2300 Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Protein concentrations were measured by the use of Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA) to normalize the fluorescence level.

RNA Analysis

Total RNA was prepared from the cells using the TriPure Isolation Reagent (Roche Diagnostics). Reverse transcription was performed with total RNA (1 µg), random hexamer (Takara-Bio, Kyoto, Japan), and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) for 60 min at 42 °C after denaturation of the RNA at 72 °C for 3 min, followed by denaturation of the enzyme at 99 °C for 5 min. First-strand cDNAs were diluted 10-fold with distilled water, and then used for quantitative PCR (qPCR) analysis. The mRNA levels of the genes were determined using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) with gene-specific primer sets (Table 1). The mRNA level of each gene was normalized to that of the 18S rRNA gene as the internal control. Data are shown as the means ± S.D. from three independent experiments.

Western Blot Analysis

Proteins were extracted as described previously [26], separated using SDS-PAGE, and transferred from the gels to a PVDF membranes (Immobilon P; Millipore, Bedford, MA, USA). The membrane was initially incubated with the primary antibody, followed by incubation with the appropriate HRP-conjugated secondary antibody. The membranes were then incubated with Pierce Western Blotting Substrate (Thermo Fisher Scientific). Immunoreactive signals were visualized by the use of Luminoimaging Analyzer LAS-3000 (Fujifilm, Tokyo, Japan), and the signals were analyzed

by Multi Gauge software (Fujifilm). The intensity of each band was normalized to that of β-actin.

Caspase-3/7 Assay

SH-SY5Y cells were incubated in 96-well white plates in DMEM containing 6-OHDA and/or fisetin. The caspase-3/7 assay was carried out by the use of a Caspase-Glo 3/7 Assay Systems (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was measured by the Lucy2 Luminescence Reader (Anthos, Salzburg, Austria).

Statistics

Two groups were compared using Student's *t*-test. One-way analysis of variance and Tukey's *post hoc* test were used to compare more than two groups with comparable variances. $p < 0.05$ was considered as statistically significant.

Results

Cell Toxicity of Fisetin in SH-SY5Y Cells

Initially, we examined the cell viability of and LDH release from SH-SY5Y cells that treated with fisetin. SH-SY5Y cells were cultured for 24 h in DMEM containing various concentrations of fisetin (0–100 µM), and cell viability and LDH release were measured. When the cells were cultured in DMEM with 50 or 100 µM of fisetin, cell viability was reduced approximately 33 and 63%, respectively, when compared with those of the vehicle-treated cells (Fig. 2b). Moreover, LDH release was elevated about 5.5- and 9.8-fold in 50 or 100 µM fisetin-treated cells, respectively, as compared with that of the vehicle-treated cells (Fig. 2c). In contrast, the cell viability remained unaffected when the cells were cultured in DMEM with 10 µM of fisetin (Fig. 2a, b). Moreover, fisetin (10 µM) did not reveal any effects on LDH release in SH-SY5Y cells (Fig. 2c). Thus, we used 10 µM fisetin in further studies.

Table 1 Nucleotide sequences of the primers used in qPCR

Gene name	Acc. no.	Forward primer	Reverse primer
HO-1	NM_002133	5′-GGCAGAGGGTGATAGAAGAGG-3′	5′-AGCTCCTGCAACTCCTCAAA-3′
NQO1	NM_000903	5′-CAGCTCACCGAGAGCCTAGT-3′	5′-GAGTGAGCCAGTACGATCAGTG-3′
NRF2	NM_001145412	5′-ACACGGTCCACAGCTCATC-3′	5′-TGCCTCCAAAGTATGTCAATCA-3′
GCLM	NM_001308253	5′-GTTGGAACAGCTGTATCAGTGG-3′	5′-CAGTCAAATCTGGTGGCATC-3′
18S rRNA	M10098	5′-GGGAGCCTGAGAAACGGC-3′	5′-GGGTCGGGAGTGGGTAATTT-3′

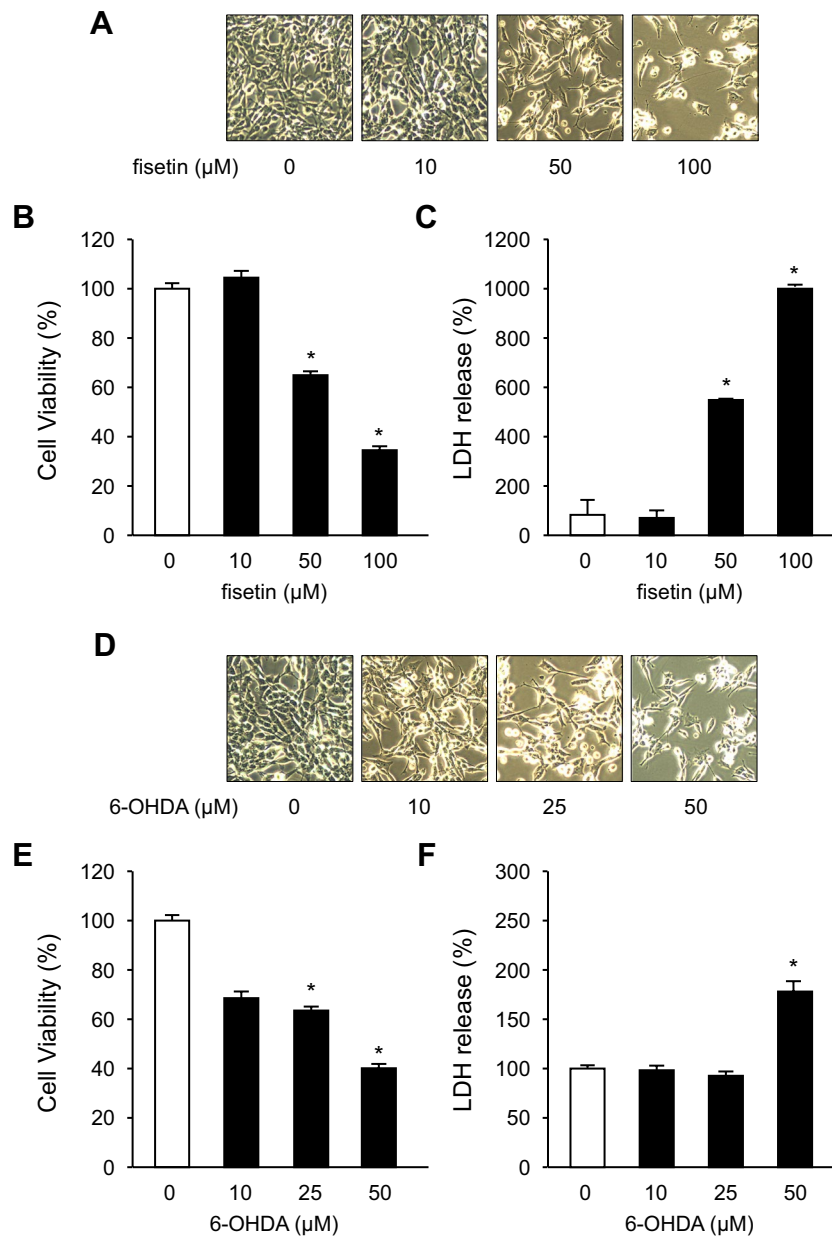*Acc. no.* Accession number of GenBank/ENA/DDBJ database

Fig. 2 Cytotoxic effects of fisetin and 6-OHDA on SH-SY5Y cells. **a** Morphology of SH-SY5Y cells treated with different concentrations of fisetin (0–100 μM) for 24 h. The data represent three independent experiments. **b** Measurement of the cytotoxic effects of fisetin in SH-SY5Y cells. Cells were cultured in DMEM containing various concentrations of fisetin (10–100 μM; black columns) for 24 h. Data are the means ± S.D. from three independent experiments. *$p < 0.01$, as compared with the vehicle control (0 μM; white column). **c** LDH release from fisetin-treated SH-SY5Y cells. The cells were treated as described in the legend of **b**. The data represent the means ± S.D from three independent experiments. *$p < 0.01$, as compared with the vehi-cle control (0 μM; white column). **d** Morphology of SH-SY5Y cells treated with 6-OHDA (0–50 μM) for 24 h. Data are the representative of three independent experiments. **e** Cell viability of 6-OHDA-treated SH-SY5Y cells. Cells were cultured in DMEM with various concentrations of 6-OHDA (10–50 μM; black columns) for 24 h. Data are the means ± S.D. from three independent experiments. *$p < 0.01$, as compared with vehicle control (0 μM; white column). **f** LDH release from 6-OHDA-treated SH-SY5Y cells. The cells were treated as described in the legend of **e**. Data are shown as the means ± S.D. from three independent experiments. *$p < 0.01$, as compared with the vehicle control (0 μM; white column)

6-OHDA-Mediated Cell Toxicity in SH-SY5Y Cells

We investigated the toxic effects of 6-OHDA in SH-SY5Y cells. The cells were cultured for 24 h in DMEM containing various concentrations of 6-OHDA (0–50 μM). Cell viability was lowered in a 6-OHDA concentration-dependent manner (Fig. 2d, e). Moreover, 6-OHDA-mediated LDH release was increased in a 6-OHDA-concentration dependent manner in

SH-SY5Y cells (Fig. 2f). Thus, we decided to use 50 µM 6-OHDA in further studies.

Decrease in 6-OHDA-Mediated Production of ROS by Fisetin

To determine the degree of 6-OHDA and fisetin-mediated production of ROS, the intracellular ROS levels were assessed by the use of DCFDA. SH-SY5Y cells were pretreated with fisetin for 3 h, and then cultured in DMEM containing 6-OHDA alone for 24 h. The cells were incubated for 30 min in the presence of DCFDA. The fluorescence in 6-OHDA-treated cells was increased as compared with the vehicle-treated cells (Fig. 3a); however, when the cells were pre-treated with fisetin prior to incubation in DMEM with 6-OHDA alone, DCFDA-mediated fluorescence was clearly decreased as compared with that seen in 6-OHDA-treated cells (Fig. 3a). Moreover, the fluorescence levels in 6-OHDA-treated cells were enhanced about 21-fold, as compared with that of the vehicle-treated cells (Fig. 3b). This increase was reduced to approximately 58% by the pretreatment with fisetin (Fig. 3b). These results indicate that pretreatment of the cells with fisetin effectively protects from 6-OHDA-induced oxidative stress in SH-SY5Y cells.

Protection Against 6-OHDA-Induced Cell Death by Fisetin

Next, we studied the protective effects of fisetin on 6-OHDA-induced death of SH-SY5Y cells. Following culture for 24 h in DMEM containing 6-OHDA, cells were damaged with changes in their morphology and number decreased (Fig. 4a); however, after pretreating the cells with several concentrations of fisetin (1–10 µM) for 3 h, 6-OHDA-mediated cell death was reduced in a fisetin concentration dependent manner (Fig. 4a). Moreover, we validated the protective effects of fisetin against 6-OHDA-induced cell death by measuring the cell viability. When SH-SY5Y cells were cultured in DMEM with 6-OHDA for 24 h, viability of the cells was lowered to about 37% of that of the vehicle-treated cells (Fig. 4b). In contrast, by pretreating the cells with various concentrations of fisetin, cell viability was elevated in a fisetin concentration-dependent manner (Fig. 4b). Moreover, 6-OHDA-enhanced LDH release was significantly lowered by pretreatment with fisetin (5–10 µM; Fig. 4c). We also measured the expression levels of the oxidative stress-related genes such as hemeoxygenase-1 (HO-1), NAD(P)H dehydrogenase quinone 1 (NQO1), NF-E2-related factor 2 (NRF2), and γ-glutamate-cysteine ligase modifier (GCLM) by qPCR. When the cells were cultured in DMEM with 6-OHDA, the mRNA levels of these genes were upregulated approximately 14.6-, 12.7-, 2.2-, and 5.6-fold, respectively, as compared with those of the vehicle treated cells
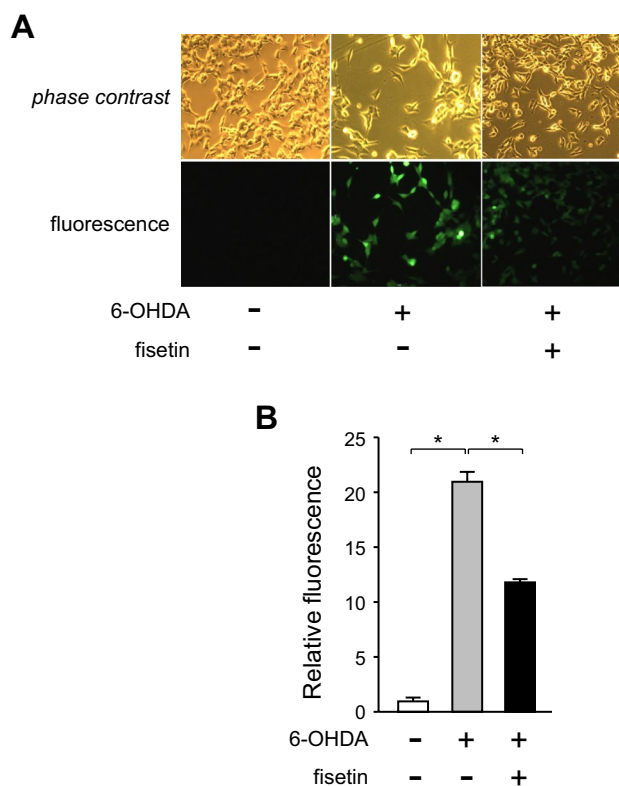
A

B

Fig. 3 Decrease in 6-OHDA-mediated intracellular ROS production by fisetin in SH-SY5Y cells. **a** Change in the intracellular ROS levels in SH-SY5Y cells by the treatment with 6-OHDA (50 µM) with or without fisetin (10 µM). Cells were pretreated with fisetin (10 µM) for 3 h, and then cultured in medium with 6-OHDA (50 µM) alone for more 24 h. Intracellular ROS levels were measured by the use of DCFDA as described in the Materials and Methods. The data are the representative of three independent experiments. **b** DCFDA-mediated fluoresce level. Cells cultured as described in the legend of **a**, were disrupted, and fluorescence levels and protein concentrations were measured. The fluorescence level was normalized using the protein concentration. Data are the means ± S.D. from three independent experiments. *$p < 0.01$, as indicated by the brackets

(Fig. 4d). Whereas, the pretreatment with several concentrations of fisetin (1–10 µM) for 3 h before culture in DMEM containing 6-OHDA alone for 24 h, results in a decrease in the expression level of the oxidative stress-related genes in fisetin-concentration dependent manner (Fig. 4d). These results suggest that fisetin protects SH-SY5Y cells from 6-OHDA-induced cell death.

Antiapoptotic Ability of Fisetin in 6-OHDA-Treated SH-SY5Y Cells

We examined the effects of fisetin on 6-OHDA-mediated cell death by measuring the levels of the proapoptotic Bax protein and the antiapoptotic Bcl-2 protein by Western blot analysis. Pretreatment with fisetin for 3 h and further culture for 24 h in DMEM did not affect the expression of Bax
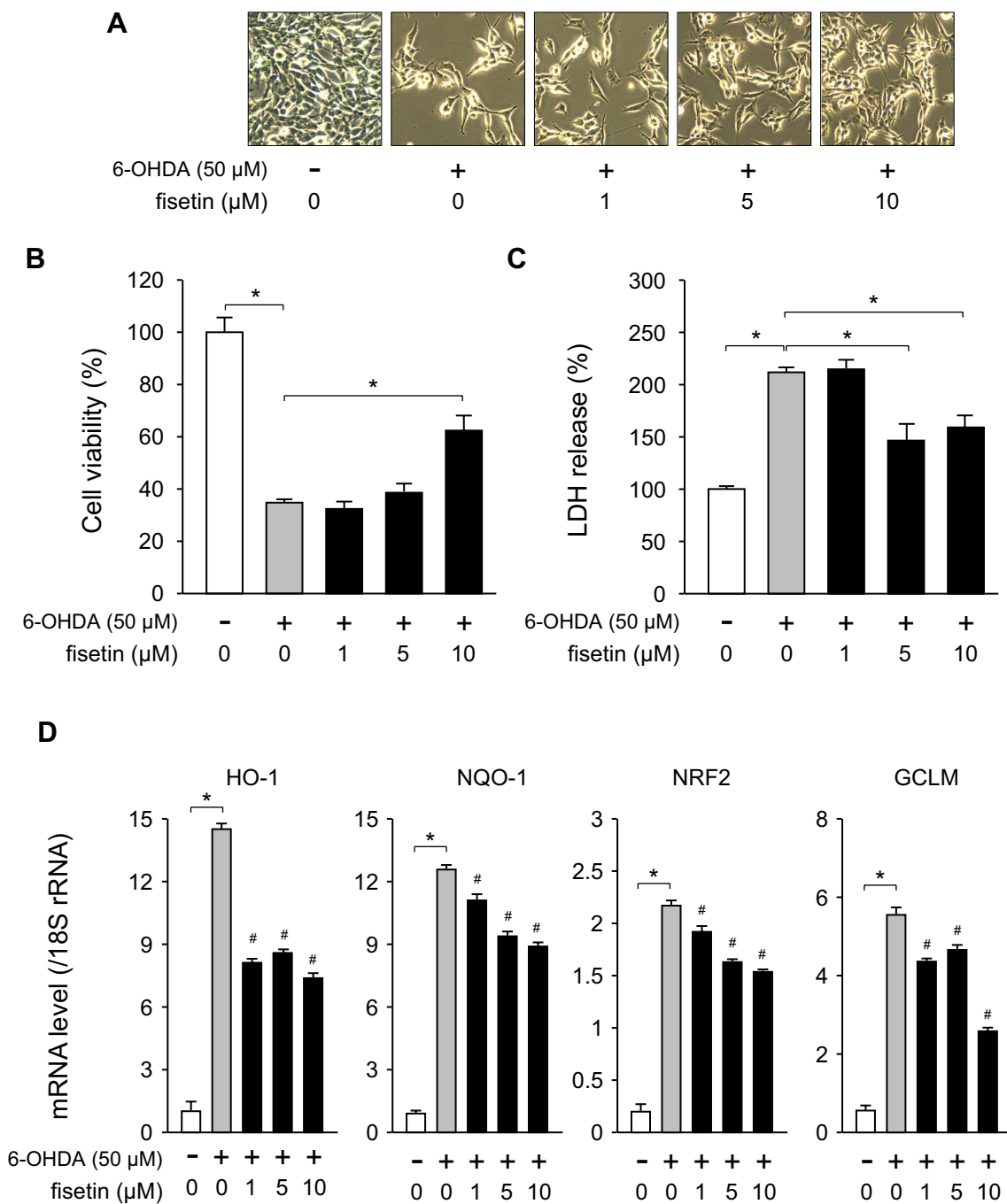
A

| 6-OHDA (50 µM) | − | + | + | + | + |
| fisetin (µM) | 0 | 0 | 1 | 5 | 10 |

B

| 6-OHDA (50 µM) | − | + | + | + | + |
| fisetin (µM) | 0 | 0 | 1 | 5 | 10 |

C

| 6-OHDA (50 µM) | − | + | + | + | + |
| fisetin (µM) | 0 | 0 | 1 | 5 | 10 |

D

Fig. 4 Protective effects of fisetin against 6-OHDA-induced cell death. **a** Morphology of SH-SY5Y cells treated with 6-OHDA (50 µM) for 24 h together with or without fisetin (0–10 µM). The data are the representative of three independent experiments. **b** Assessment of the protective effects of fisetin on the viability of 6-OHDA-treated SH-SY5Y cells. Cells were pretreated with various concentrations of fisetin (0–10 µM) for 3 h, and were then further cultured in DMEM containing 6-OHDA (50 µM) alone for 24 h. Cell viability was measured by the WST assay. Data are the means ± S.D. from three independent experiments. *$p < 0.01$, as indicated by the brackets. **c** Measurement of LDH release in fisetin (0–10 µM) and/or 6-OHDA (50 µM)-treated SH-SY5Y cells. The cells were cultured as described in the legend of **b**. The data represent the means ± S.D from three independent experiments. *$p < 0.01$, as indicated by the brackets. **d** Changes in the transcription levels of the oxidative stress-associated genes in SH-SY5Y cells. The cells were cultured as described in the legend of **b**, and the mRNA levels were measured by qPCR. The data represent the means ± S.D from three independent experiments. *$p < 0.01$, as indicated by the brackets. #$p < 0.01$, as compared with that of 6-OHDA-treated cells

and Bcl-2 (Fig. 5a, b). Treatment with 6-OHDA slightly increased the Bax/Bcl-2 ratio (Fig. 5c, d). In contrast, when the cells were cultured in DMEM with 6-OHDA after pre-treating with fisetin, the Bax/Bcl-2 ratio was reduced approximately 24% of that of the 6-OHDA-treated cells (Fig. 5c, d). These results mean that pretreatment with fisetin has the potential to reduce 6-OHDA-mediated progression of apoptosis in SH-SY5Y cells.

To further obtain evidence that fisetin protects against 6-OHDA-mediated apoptosis in SH-SY5Y cells, at first we investigated the effects of fisetin in the activation of caspase cascade, which is important in the progression of apoptosis. Pretreatment with fisetin and further culture for 24 h in

DMEM did not show any effects in the activation of caspase-3 and caspase-9 (Fig. 6a). The caspase-3/7 activity in fisetin-treated SH-SY5Y cells by the use of the Caspase-Glo 3/7 Assay System. When the cells were pretreated with fisetin and further cultured in DMEM, caspase-3/7 activity was not changed (Fig. 6b).

When SH-SY5Y cells were incubated in DMEM with 6-OHDA for 24 h after pretreatment in DMEM for 3 h, cleaved caspase-3 and caspase-9 levels were increased, as compared with those of the vehicle-treated cells (Fig. 6c). In contrast, when the cells were pretreated with fisetin before incubation in DMEM containing 6-OHDA alone, the levels of both were clearly decreased (Fig. 6c). In addition,
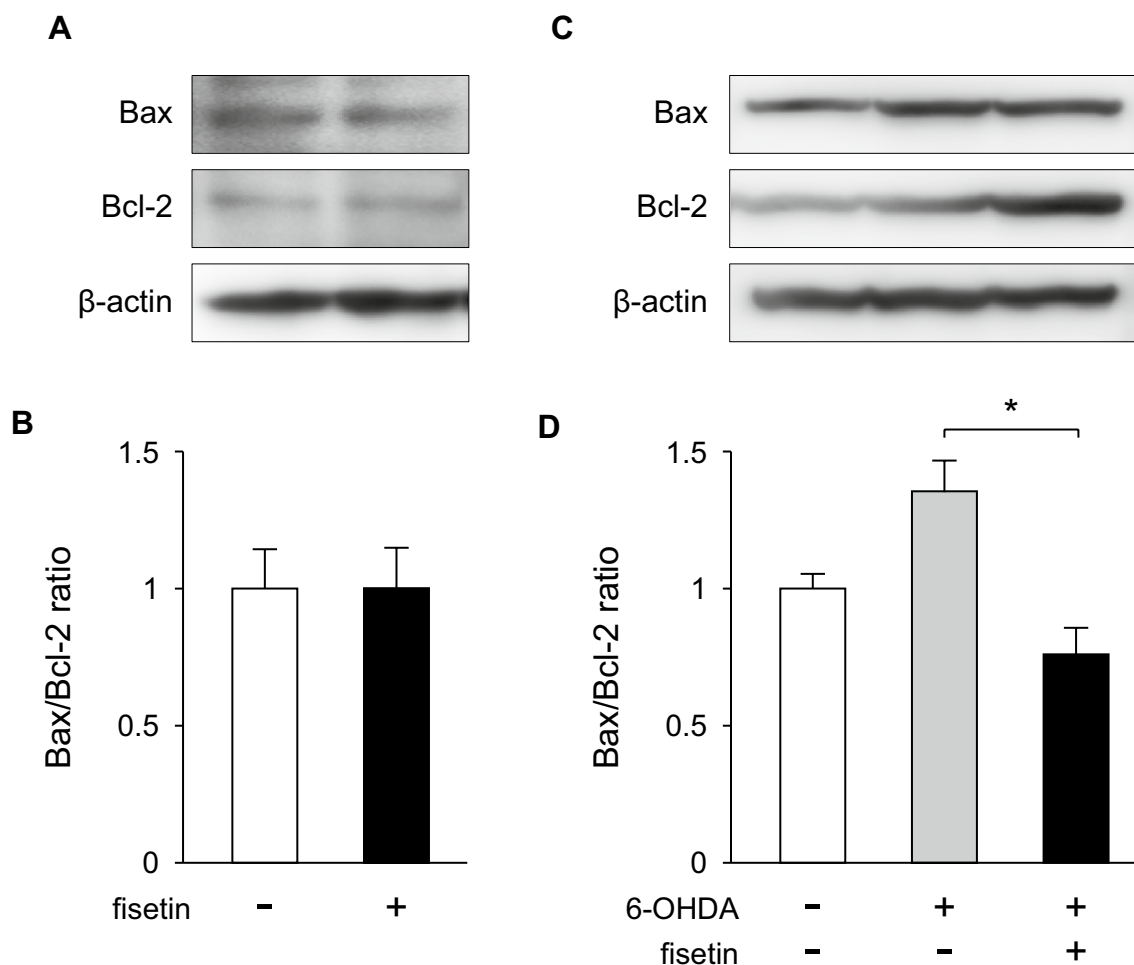

Fig. 5 Changes in the expression of Bax and Bcl-2 in fisetin/6-OHDA-treated SH-SY5Y cells. **a** Expression of Bax and Bcl-2 in the cells pretreated with fisetin. SH-SY5Y cells were pretreated with fisetin (10 µM) for 3 h, and further cultured in DMEM without fisetin and 6-OHDA for 24 h. Crude cell extracts were prepared and protein samples (15 µg) were loaded into each lane for SDS-PAGE and Western blot analysis by the use of anti-Bax, anti-Bcl-2, and anti-β-actin antibodies. Data are the representative from three independent experiments. **b** Band intensities of the results of **a**. Band intensities were analyzed by using MultiGauge software, and the Bax/Bcl-2 ratio is shown. The data represent the means ± S.D from three independent experiments. **c** Expression of Bax and Bcl-2 in fisetin/6-OHDA-treated SH-SY5Y cells. The cells were pretreated with fisetin (10 µM) for 3 h, and further cultured in medium containing 6-OHDA (50 µM) for 24 h. Crude protein (15 µg/lane) was used in Western blot analysis. Data are the representative from three independent experiments. **d** Band intensities of the results of **c**. The data represent the means ± S.D from three independent experiments. *$p < 0.01$, as indicated by the bracket

A

cleaved caspase-3

cleaved caspase-9

β-actin

| fisetin | − | + |

C

cleaved caspase-3

cleaved caspase-9

β-actin

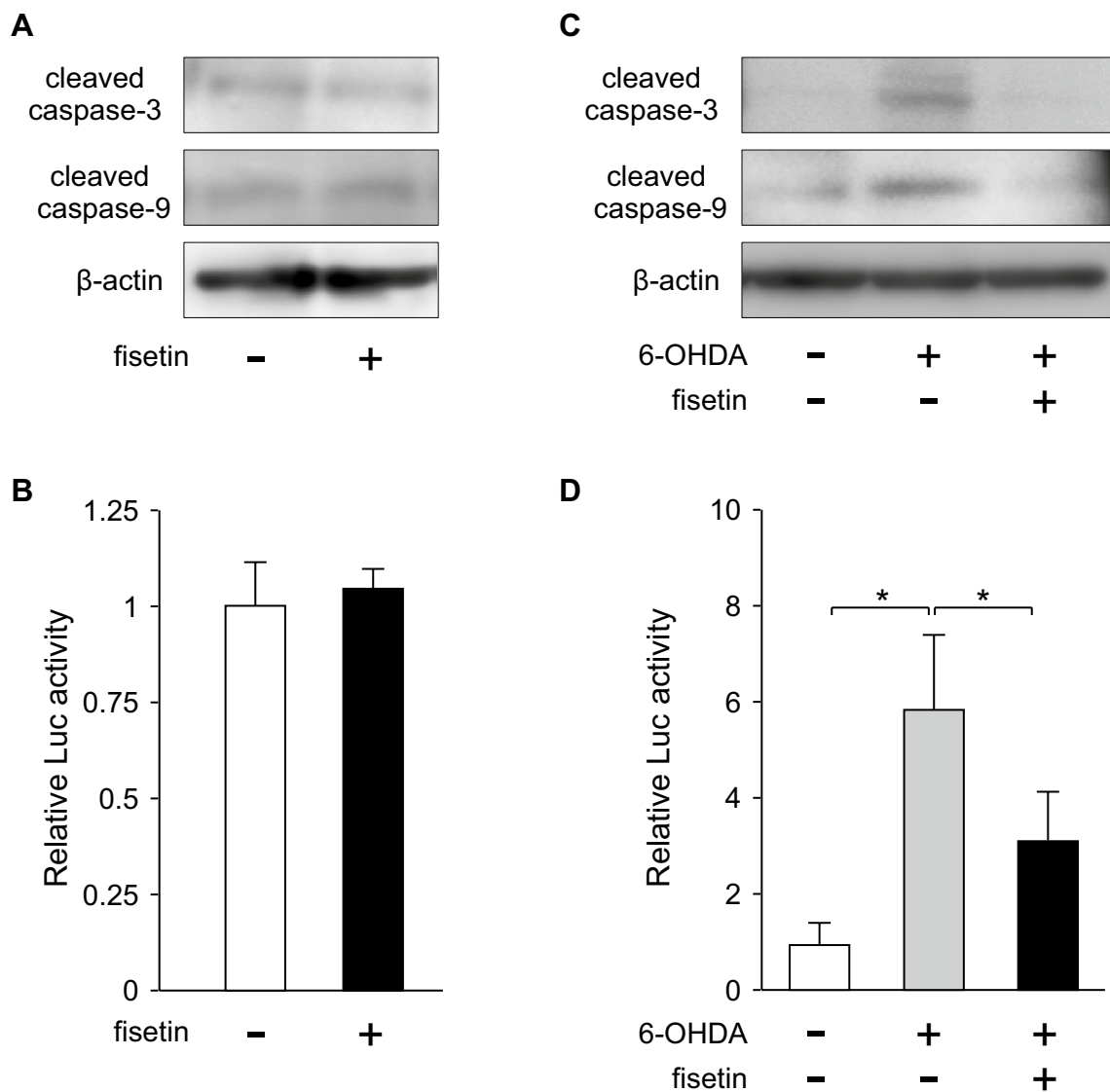| 6-OHDA | − | + | + |
| fisetin | − | − | + |

B

D

Fig. 6 Protective effects of fisetin against 6-OHDA-induced apoptosis in SH-SY5Y cells. **a** Levels of cleaved caspase-3 and caspase-9 in SH-SY5Y cells pretreated with fisetin. SH-SY5Y cells were pretreated with fisetin (10 μM) for 3 h, and further cultured in DMEM without fisetin and 6-OHDA for 24 h. Crude cell extracts were prepared and protein samples (20 μg) were loaded into each lane for SDS-PAGE and Western blot analysis by the use of anti-cleaved caspase-3, anti-cleaved caspase-9, and anti-β-actin antibodies. Data are the representative from three independent experiments. **b** Caspase-3/7 activity. SH-SY5Y cells were cultured as described in the legend of **a**. Activity of caspase-3/7 was measured by the use of the Caspase-Glo

3/7 Assay Systems. Data are the means ± S.D. from three independent experiments. **c** SH-SY5Y cells were pretreated with fisetin (10 μM) for 3 h, and further cultured in medium containing 6-OHDA (50 μM) for 24 h. Crude protein (20 μg/lane) was used in Western blot analysis. Data are the representative from three independent experiments. **d** Inhibition of 6-OHDA-activated caspase-3/7 by fisetin in SH-SY5Y cells. The cells were cultured as described in the legend of Fig. 5a. Activity of caspase-3/7 was measured by the use of the Caspase-Glo 3/7 Assay Systems. Data are the means ± S.D. from three independent experiments. *$p < 0.01$, as indicated by the brackets

caspase-3/7 activity was elevated about 5.6-fold by the treatment with 6-OHDA, as compared with that of the vehicle-treated cells (Fig. 6d). The elevated caspase-3/7 activity was reduced to about 48% by the pretreatment with fisetin (Fig. 6d). These results reveal that 6-OHDA-induced activation of the caspase cascade was inhibited by pretreatment with fisetin in SH-SY5Y cells.

Suppression of 6-OHDA-Mediated Cell Death by Fisetin Via Akt Signaling

Phosphoinositide 3-kinase (PI3K) and its downstream Akt are known to inhibit apoptosis and promote cell survival [27]. To investigate the involvement of the Akt signaling in the suppression of 6-OHDA-mediated cell death by fisetin,

we investigated the activation (phosphorylation) of Akt in 6-OHDA- and/or fisetin-treated cells. Since phosphorylation of Akt is required for its activation, we investigated by Western blot analysis.

Firstly, we pretreated the cells in DMEM with fisetin for 3 h, and further cultured in DMEM for 24 h. Phosphorylation level of Akt was enhanced approximately 2.1-fold by the pretreatment with fisetin (Fig. 7a, b). Moreover, this increased phosphorylation level was almost unchanged after 24 h (Fig. 7a, b). Furthermore, pretreatment with fisetin before the cells underwent 6-OHDA treatment, phosphorylation of Akt was increased about 1.9-fold, as compared with 6-OHDA-treated cells (Fig. 7c, d).

To further confirm the involvement of Akt signaling, 6-OHDA- and/or fisetin-treated cells were we co-treated with Akt inhibitor (Akti). Akti negated the fisetin-mediated suppression of 6-OHDA-induced cell death (Fig. 7e, f). However, Akti itself did not affect the viability of SH-SY5Y cells (Fig. 7e, f). These results reveal that fisetin-mediated suppression of 6-OHDA-induced cell death occurred through activation of PI3K-Akt signaling in SH-SY5Y cells.

Discussion

The loss of dopaminergic neurons in the substantia nigra is thought to be a direct cause of neurodegeneration in PD [1]. Oxidative stress is a major cause of the neurodegenerative diseases such as PD by inducing cell death via mitochondrial dysfunction, DNA mutation, and so on [28]. 6-OHDA is widely used to mimic PD through ROS-mediated oxidative stress [5]. Moreover, 6-OHDA-treated cells are used to model of PD and to evaluate therapeutic agents and bioactive compounds as potential treatments for the disease. Thus, inhibiting the production of ROS is an effective strategy to lower the oxidative stress-mediated cell death, and to prevent the development of its associated diseases. Previously, biological effects of fisetin in brain and neuronal cells have been identified in vivo [22–25] as well as in vitro [19–21]. However, the protective mechanism of ROS-mediated cell death by fisetin in neuronal cells has not been well elucidated. In this study, we found the molecular mechanism of protective effects of fisetin against 6-OHDA-induced cell death in human neuronal SH-SY5Y cells.

To date, natural bioactive compounds such as flavonoids have been shown neuroprotective effects in neuronal cells [29–31]. Flavonoids are polyphenolic compounds that are widely found in nature, and are categorized into flavans, flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones by their chemical structure. Fisetin, a flavonoid possesses a range of biological activities including anticancer, antimicrobial, anti-inflammatory, antioxidant anti-adipogenic effects [15, 32–36]. Pretreatment with fisetin

for 3 h decreased 6-OHDA-induced cell death in SH-SY5Y cells (Fig. 4) through repression of ROS production (Fig. 3). Moreover, fisetin induced the expression of antiapoptotic Bcl-2 protein (Fig. 5c, d).

Pretreatment with fisetin is critical to protect against 6-OHDA-mediated ROS production. Phosphorylated Akt level was increased in this pretreatment, and this phosphorylation level was maintained for next 24 h (Fig. 7a, b). Moreover, this pretreatment did not affect to the expression of Bax and Bcl-2 (Fig. 5a, b) and activation of caspase cascade (Fig. 6a, b). Thus, pretreatment with fisetin is critical to exert antioxidative effects via direct scavenging of ROS through PI3K/Akt signaling in neuronal cells.

10 μM fisetin used in this study did not show any cell toxicity (Fig. 2a, b). However, higher concentration (> 50 μM) of fisetin decreased cell viability (Fig. 2b). We think that such high concentration of fisetin may inhibit PI3K/Akt signaling through induction of phosphatase and tensin homolog (PTEN) [37]. Further analysis should be needed to identify this question.

Apoptosis is the process of cell death through characteristic phenomena such as activation of the caspase cascade, DNA fragmentation, and nuclear condensation. In mammalian cells, the caspase cascade is activated by the mitochondrial cytochrome c, which regulates energy metabolism and apoptosis [38]. Antiapoptotic Bcl-2 protein inhibits the release of cytochrome c [39], and is known to protect the neuronal cells from oxidative stress and apoptosis in PD [40]. In contrast, Bax, a proapoptotic protein enhances the progression of apoptosis through affecting to the membrane permeability [39]. In many cells, activation of apoptotic signaling is determined by the molar ratio between proapoptotic (e.g., Bax, Bak, Bcl-xS, and Bad) and antiapoptotic (e.g., Bcl-2 and Bcl-xL) proteins of the Bcl-2 family [41]. Our present results reveal that 6-OHDA-mediated elevation of the Bax/Bcl-2 ratio was suppressed by the treatment with fisetin in SH-SY5Y cells (Fig. 5c, d). Moreover, 6-OHDA-mediated activation of the caspase cascade was suppressed by fisetin (Fig. 6c, d).

We investigated the molecular mechanism of fisetin-mediated protection of 6-OHDA-indcued cell death in neuronal cells. In previous study, the ERK signaling is involved in the fisetin-mediated effects in neuronal cells [19, 22]. Moreover, fisetin-suppressed hypoxia-induced cell death was negated by inhibitor of MEK1/2, p38 MAPK or PI3K [42]. In this study, we found the involvement of the PI3K-Akt signaling in the protection of 6-OHDA-induced cell death by fisetin in human neuronal cells. Akt, also known as protein kinase B, is a downstream of PI3K, and is crucial in cell growth for mediating neuronal survival against oxidative stress [43, 44]. Activation of PI3K-Akt signaling inhibits proapoptotic proteins such as caspase-3 and caspase-9 [43], resulting in suppression of cell death. Fisetin-activated Akt
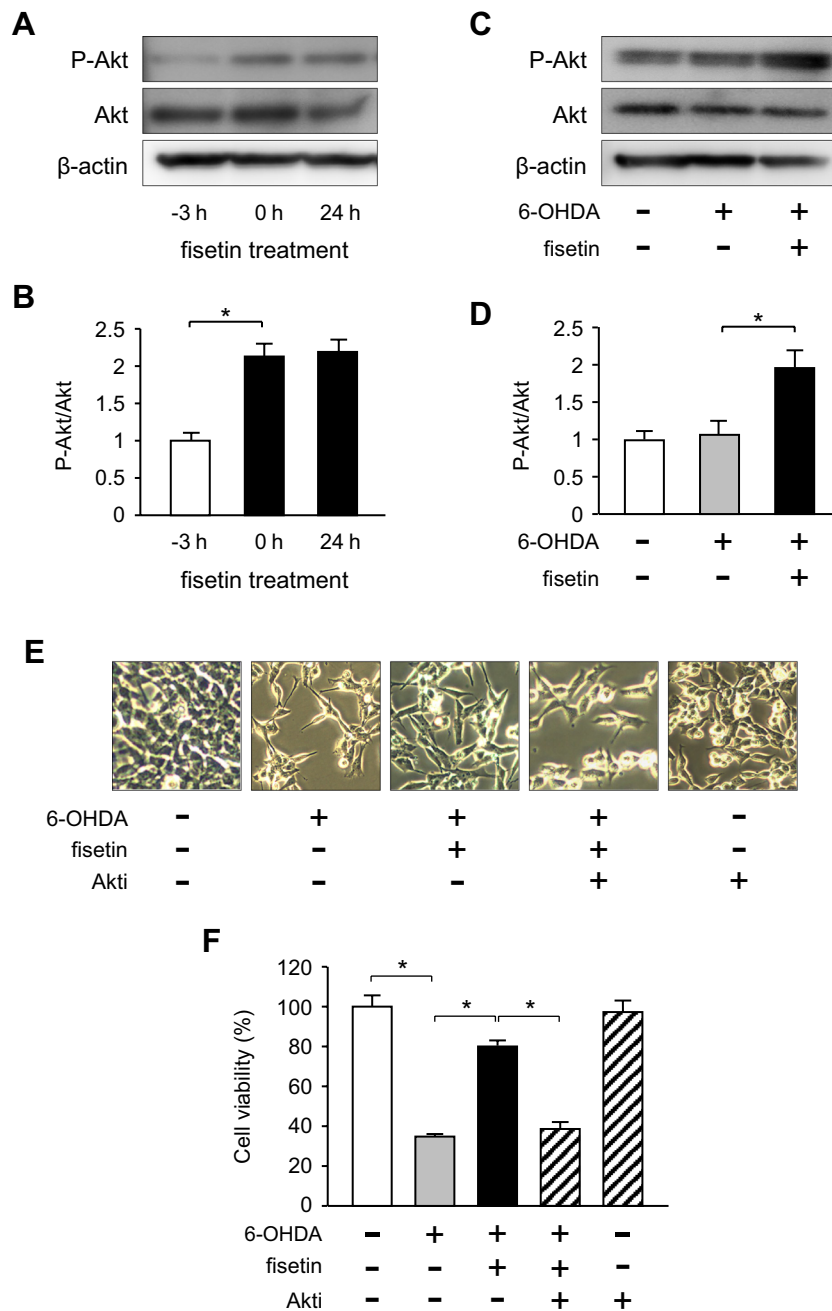
Fig. 7 Involvement of PI3K-Akt signaling in suppression of 6-OHDA-induced cell death by fisetin. **a** Activation of Akt by fisetin in SH-SY5Y cells. Cells (-3 h) were pretreated with fisetin (10 μM) for 3 h (0 h), and further cultured in DMEM for further 24 h (24 h). Protein samples (15 μg) were loaded into each lane for SDS-PAGE and Western blot analysis. β-actin was also detected as an internal control. The data are the representative of three independent experiments. **b** Changes in band intensity for phospho(P)-Akt and Akt from the results of **a**. P-Akt/Akt ratio is shown. Data are means ± S.D. from three independent experiments. *$p < 0.01$, as indicated by the bracket. **c** Activation of 6-OHDA-supressed Akt by fisetin in SH-SY5Y cells. Cells were pretreated with fisetin (10 μM) for 3 h, and further cultured in DMEM containing 6-OHDA (50 μM) alone for further 24 h. Protein samples (15 μg/lane) were loaded in Western blot analysis.

β-actin was also detected as an internal control. The data are the representative of three independent experiments. **d** Changes in band intensity for phospho(P)-Akt and Akt from the results of **c**. P-Akt/Akt ratio is shown. Data are means ± S.D. from three independent experiments. *$p < 0.01$, as indicated by the bracket. **e** Morphology of fisetin/6-OHDA and Akt inhibitor (Akti)-treated SH-SY5Y cells. The cells were pretreated with fisetin (10 μM) and Akti (10 μM) for 3 h, and further cultured in medium containing 6-OHDA (50 μM) and/or Akti for 24 h. The data are the representative of three independent experiments. **f** Cell viability of fisetin/6-OHDA and Akti-treated SH-SY5Y cells. Cells were cultured as described in the legend of **e**. Data are the means ± S.D. from three independent experiments. *$p < 0.01$, as indicated by the brackets

suppressed 6-OHDA-mediated cell death, and this activation was negated by the co-treatment with Akt inhibitor (Fig. 7).

Therefore, fisetin suppresses 6-OHDA-induced cell death through activation of the PI3K-Akt signaling in human neuronal SH-SY5Y cells.

In summary, 6-OHDA-induced cell death in human neuronal SH-SY5Y cells occurs by the generation of ROS. 6-OHDA-mediated cell death was suppressed by a flavonoid fisetin via activation of PI3K-Akt signaling. This is the first report that the PI3K-Akt signaling is included in the protection of ROS-induced cell death by fisetin in neuronal cells.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Liu Z, Zhou T, Ziegler AC, Dimitrion P, Zuo L (2017) Oxidative stress in neurodegenerative diseases: from molecular mechanisms to clinical applications. Oxid Med Cell Longev 2017:2525967
2. Farrer MJ (2006) Genetics of Parkinson disease: paradigm shifts and future prospects. Nat Rev Genet 7:306–318
3. Bekris LM, Mata IF, Zabetian CP (2010) The genetics of Parkinson disease. J Geriatr Psychiatry Neurol 23:228–242
4. Dias V, Junn E, Mouradian MM (2013) The role of oxidative stress in Parkinson's disease. J Parkinsons Dis 3:461–491
5. Lehmensiek V, Tan EM, Liebau S, Lenk T, Zettlmeisl H, Schwarz J, Storch A (2006) Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant alpha-synucleins related to Parkinson's disease. Neurochem Int 48:329–340
6. Yuan WJ, Yasuhara T, Shingo T, Muraoka K, Agari T, Kameda M, Uozumi T, Tajiri N, Morimoto T, Jing M, Baba T, Wang F, Leung H, Matsui T, Miyoshi Y, Date I (2008) Neuroprotective effects of edaravone-administration on 6-OHDA-treated dopaminergic neurons. BMC Neurosci 9:75
7. Henning J, Strauss U, Wree A, Gimsa J, Rolfs A, Benecke R, Gimsa U (2008) Differential astroglial activation in 6-hydroxydopamine models of Parkinson's disease. Neurosci Res 62:246–253
8. Radi E, Formichi P, Battisti C, Federico A (2014) Apoptosis and oxidative stress in neurodegenerative diseases. J Alzheimers Dis 42(Suppl 3):S125–S152
9. Filograna R, Beltramini M, Bubacco L, Bisaglia M (2016) Antioxidants in parkinson's disease therapy: a critical point of view. Curr Neuropharmacol 14:260–271
10. Connolly BS, Lang AE (2014) Pharmacological treatment of Parkinson disease: a review. JAMA 311:1670–1683
11. Hughes KC, Gao X, Kim IY, Rimm EB, Wang M, Weisskopf MG, Schwarzschild MA, Ascherio A (2016) Intake of antioxidant vitamins and risk of Parkinson's disease. Mov Disord 31:1909–1914
12. Mazo NA, Echeverria V, Cabezas R, Avila-Rodriguez M, Aliev G, Leszek J, Barreto GE (2017) Medicinal plants as protective strategies against Parkinson's disease. Curr Pharm Des 23:4180–4188
13. Di Carlo G, Mascolo N, Izzo AA, Capasso F (1999) Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sci 65:337–353
14. Kawser Hossain M, Abdal Dayem A, Han J, Yin Y, Kim K, Kumar Saha S, Yang GM, Choi HY, Cho SG (2016) Molecular

15. Funakoshi-Tago M, Nakamura K, Tago K, Mashino T, Kasahara T (2011) Anti-inflammatory activity of structurally related flavonoids, apigenin, luteolin and fisetin. Int Immunopharmacol 11:1150–1159
16. Birt DF, Hendrich S, Wang W (2001) Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther 90:157–177
17. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418–425
18. Shukla S, Gupta S (2006) Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther 5:843–852
19. Sagara Y, Vanhnasy J, Maher P (2004) Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. J Neurochem 90:1144–1155
20. Yen JH, Wu PS, Chen SF, Wu MJ (2017) Fisetin protects PC12 cells from tunicamycin-mediated cell death via reactive oxygen species scavenging and modulation of Nrf2-driven gene expression, SIRT1 and MAPK signaling in PC12 cells. Int J Mol Sci 18(4):852
21. Zheng LT, Ock J, Kwon BM, Suk K (2008) Suppressive effects of flavonoid fisetin on lipopolysaccharide-induced microglial activation and neurotoxicity. Int Immunopharmacol 8:484–494
22. Maher P, Akaishi T, Abe K (2006) Flavonoid fisetin promotes ERK-dependent long-term potentiation and enhances memory. Proc Natl Acad Sci USA 103:16568–16573
23. Prakash D, Sudhandiran G (2015) Dietary flavonoid fisetin regulates aluminium chloride-induced neuronal apoptosis in cortex and hippocampus of mice brain. J Nutr Biochem 26:1527–1539
24. Patel MY, Panchal HV, Ghribi O, Benzeroual KE (2012) The neuroprotective effect of fisetin in the MPTP model of Parkinson's disease. J Parkinsons Dis 2:287–302
25. Zbarsky V, Datla KP, Parkar S, Rai DK, Aruoma OI, Dexter DT (2005) Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease. Free Radic Res 39:1119–1125
26. Nakao Y, Yoshihara H, Fujimori K (2016) Suppression of very early stage of adipogenesis by baicalein, a plant-derived flavonoid through reduced Akt-C/EBPα-GLUT4 signaling-mediated glucose uptake in 3T3-L1 adipocytes. PLoS ONE 11:e0163640
27. Will M, Qin AC, Toy W, Yao Z, Rodrik-Outmezguine V, Schneider C, Huang X, Monian P, Jiang X, de Stanchina E, Baselga J, Liu N, Chandarlapaty S, Rosen N (2014) Rapid induction of apoptosis by PI3K inhibitors is dependent upon their transient inhibition of RAS-ERK signaling. Cancer Discov 4:334–347
28. Crotty GF, Ascherio A, Schwarzschild MA (2017) Targeting urate to reduce oxidative stress in Parkinson disease. Exp Neurol 298:210–224
29. Jager AK, Saaby L (2011) Flavonoids and the CNS. Molecules 16:1471–1485
30. Diniz TC, Silva JC, de Lima-Saraiva SR, Ribeiro FP, Pacheco AG, de Freitas RM, Quintans-Junior LJ, Quintans Jde S, Mendes RL, Almeida JR (2015) The role of flavonoids on oxidative stress in epilepsy. Oxid Med Cell Longev 2015:171756
31. Wang ZY, Liu JY, Yang CB, Malampati S, Huang YY, Li MX, Li M, Song JX (2017) Neuroprotective natural products for the treatment of parkinson's disease by targeting the autophagy-lysosome pathway: a systematic review. Phytother Res 31:1119–1127
32. Gabor M, Eperjessy E (1966) Antibacterial effect of fisetin and fisetinidin. Nature 212:1273

33. Adhami VM, Syed DN, Khan N, Mukhtar H (2012) Dietary flavonoid fisetin: a novel dual inhibitor of PI3K/Akt and mTOR for prostate cancer management. Biochem Pharmacol 84:1277–1281

34. Watanabe M, Hisatake M, Fujimori K (2015) Fisetin suppresses lipid accumulation in mouse adipocytic 3T3-L1 cells by repressing GLUT4-mediated glucose uptake through inhibition of mTOR-C/EBPα signaling. J Agric Food Chem 63:4979–4987

35. Currais A, Farrokhi C, Dargusch R, Armando A, Quehenberger O, Schubert D, Maher P (2017) Fisetin reduces the impact of aging on behavior and physiology in the rapidly aging SAMP8 mouse. J Gerontol A Biol Sci Med Sci. https://doi.org/10.1093/gerona/glx104

36. Currais A, Prior M, Dargusch R, Armando A, Ehren J, Schubert D, Quehenberger O, Maher P (2014) Modulation of p25 and inflammatory pathways by fisetin maintains cognitive function in Alzheimer's disease transgenic mice. Aging Cell 13:379–390

37. Khan N, Afaq F, Khusro FH, Mustafa Adhami V, Suh Y, Mukhtar H (2012) Dual inhibition of phosphatidylinositol 3-kinase/Akt and mammalian target of rapamycin signaling in human nonsmall cell lung cancer cells by a dietary flavonoid fisetin. Int J Cancer 130:1695–1705

38. Cai J, Yang J, Jones DP (1998) Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta 1366:139–149

39. Vander Heiden MG, Thompson CB (1999) Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1:E209–E216

40. Tortosa A, Lopez E, Ferrer I (1997) Bcl-2 and Bax proteins in lewy bodies from patients with Parkinson's disease and diffuse lewy body disease. Neurosci Lett 238:78–80

41. Raisova M, Hossini AM, Eberle J, Riebeling C, Wieder T, Sturm I, Daniel PT, Orfanos CE, Geilen CC (2001) The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. J Invest Dermatol 117:333–340

42. Chen PY, Ho YR, Wu MJ, Huang SP, Chen PK, Tai MH, Ho CT, Yen JH (2015) Cytoprotective effects of fisetin against hypoxia-induced cell death in PC12 cells. Food Funct 6:287–296

43. Martelli AM, Faenza I, Billi AM, Manzoli L, Evangelisti C, Fala F, Cocco L (2006) Intranuclear 3′-phosphoinositide metabolism and Akt signaling: new mechanisms for tumorigenesis and protection against apoptosis? Cell Signal 18:1101–1107

44. Brader S, Eccles SA (2004) Phosphoinositide 3-kinase signalling pathways in tumor progression, invasion and angiogenesis. Tumori 90:2–8