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Neurotrophic and Cytoprotective Action of Luteolin in PC12 Cells through ERK-Dependent Induction of Nrf2-Driven HO-1 Expression

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Luteolin (3′,4′,5,7-tetrahydroxyflavone), a food-derived flavonoid, has been reported to possess antioxidant, anti-inflammatory, and anticancer activities. In this work, we assessed whether luteolin has neurotrophic activity, namely, the ability to induce neurite outgrowth and to attenuate serum withdrawal-induced cytotoxicity in PC12 cells. Our results show that luteolin significantly induced neurite outgrowth along with increased expression of the differentiation marker, growth-associated protein-43 (GAP-43), in PC12 cells dose-dependently. Incubation of serum-deprived PC12 cells with luteolin prevented apoptosis, increased the expression of heme oxygenase-1 (HO-1) mRNA and protein levels, and enhanced the binding of nuclear factor E2-related factor 2 (Nrf2) to antioxidant response element (ARE), which works as an enhancer sequence in the HO-1 promoter. Addition of zinc protoporphyrin (Znpp), a selective HO-1 competitive inhibitor, significantly reduced the cytoprotective ability of luteolin, indicating the vital role of HO-1. Luteolin also persistently activated extracellular signal-regulated protein kinase 1/2 (ERK1/2); while the addition of U0126, a pharmacological MEK/ERK inhibitor, attenuated luteolin-induced Nrf2 binding activity, HO-1 expression, cytoprotective effect, and neurite outgrowth. Taken together, the above findings suggest that luteolin induces neurite outgrowth and augments cellular antioxidant defense capacity, at least in part, through the activation of the ERK signaling pathway.

KEYWORDS: Luteolin; PC12 cells; HO-1; Nrf2; ARE; ERK

INTRODUCTION

Neurotrophic factors play important roles in the promotion of the development, survival, and functional maintenance of neuronal cells. The levels of neurotrophic factors are changed in the pathophysiology of a wide variety of neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (*1*). Nerve growth factor (NGF), one member of the neurotrophins, has been reported as the most efficacious neurotrophic factor acting on basal forebrain cholinergic neurons (BFCNs) and suggested as a potential therapeutic agent for protection against the degeneration of cholinergic neurons in patients with AD (*2*). However, NGF is a polypeptide, and its application on peripheral administration is severely restricted by the difficulties in passing across the blood−brain barrier (BBB) and delivery to the brain. As a result, identification of small molecules that can mimic the function of NGF and substitute for its clinical use serves as an alternative approach (*3*).

PC12 cells, a rat pheochromocytoma cell line, are a widely used model system for studies of molecular mechanisms underlying neuronal cell differentiation and cell death following limited supplies of neurotrophic factor, and to identify the molecules that can maintain the survival of nerve cells in growth factor-deprived culture medium (*4*). It has been characterized that NGF could promote neurite outgrowth and prevent cell death in PC12 cells under serum-withdrawal condition (*5*, *6*).

Flavonoids are a variety of polyphenolic compounds widely distributed in plants and are regularly consumed in the diet. Several physiological benefits have been attributed to flavonoids, including protection from inflammation, cardiovascular heart disease, and cancer (*7*). Recent studies of polyphenolics further suggested that flavonoid compounds might be useful in the prevention of neurodegenrative disorders (*8*, *9*). Flavonoid compounds such as epigallocatechin-3-gallate (EGCG) (*10*), kaempferol (*11*), and apigenin (*12*) have been demonstrated as neuroprotective molecules to promote neuronal cell differentiation or protect neuronal cells against serum deprivation-induced oxidative stress.

Luteolin (3′,4′,5,7-tetrahydroxyflavone, **Figure 1**) is a flavonoid existing mainly in the glycosylated form in many types of plants including fruits, vegetables, and medicinal herbs and is considered as the major antioxidant, anti-inflammation, and anticancer constituent (*13*, *14*). Luteolin could attenuate hydrogen peroxide (H_2O_2)- (*15*) and amyloid β (Aβ) protein-induced (*16*) neurotoxicity in vitro. Tsai et al. reported that luteolin could

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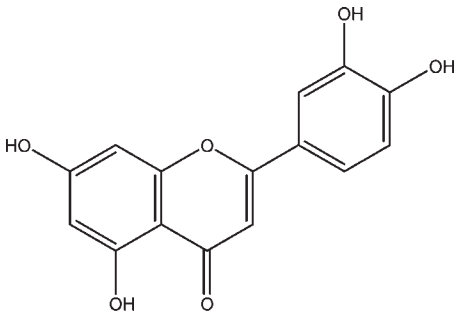

Luteolin

Figure 1. Chemical structure of luteolin.

permeate through the blood−brain barrier in vivo and could attenuate scopolamine-induced amnesia in rats (*17*). The above reports reveal that luteolin possesses neuroprotective effects; however, the detailed molecular mechanism regarding its possible neurogenic action to promote neuronal cell differentiation and against serum-deprivation apoptosis remain limited. In the present study, we demonstrate that luteolin induces neurite outgrowth and augments cellular antioxidant defense capacity through the activation of ERK signal pathways that leads to Nrf2 activation and HO-1 expression in rat pheochromocytoma (PC12) cells.

MATERIALS AND METHODS

Chemicals. Luteolin, poly-L-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI), and zinc protoporphyrin (Znpp) as well as other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated. 1,4-Diamino-2,3-dicyano-1,4-bis-(2-aminophenylthio)-butadiene (U0126), a selective and potent inhibitor of MEK activity and activation of ERK1/2, was purchased from Promega (Madison, WI, USA).

Cell Culture. The rat adrenal pheochromocytoma cell line, PC12, was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in RPMI-1640 medium (Sigma-Aldrich), which contains 2 mM glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate, supplemented with 10% heat-inactivated horse serum (HS, Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS, Biological Industries, Kibbutz Haemek, Israel) in a 5% CO_2 incubator at 37 °C.

Analysis of Neurite Outgrowth of PC12 Cells. PC12 cells (2×10^5/mL) were seeded on poly-L-lysine-coated 6-well plates in normal serum medium for 24 h. The freshly made low serum RPMI medium (1% HS and 0.5% FBS) was replaced prior to exposure to the indicated reagent or DMSO vehicle (final concentration of 0.1%). Neurite outgrowth was quantified after an additional 72 h of incubation as the proportion of cells with neurites greater than or equal to the length of one cell body. These determinations were made with an Olympus IX71 inverted microscope using phase-contrast objectives. The number of neurite-bearing cells was counted in at least five randomly selected microscopic fields and expressed as a percentage of the total cells in the field. The neurite length was also determined for all identified neurite-bearing cells in a field by tracing the longest length of neurite per cell using Image J software (NIH Image software). Each experiment was conducted in triplicate.

Analysis of Serum Deprivation-Induced PC12 Cell Death. PC12 cells (2×10^5/mL) were seeded in 24-well plates and cultured in serum-free RPMI-1640 medium supplemented with the indicated reagent or DMSO vehicle (final concentration of 0.1%) for 48 h. Cell viability was measured by MTT assay as described below.

Cell Viability. Cell viability was measured by the mitochondrial-dependent reduction of MTT to purple formazan. Briefly, cells were incubated with MTT solution (1 mg/mL final concentration) for 4 h at 37 °C followed by centrifugation at 8,000*g* for 4 min. The medium was carefully removed by aspiration, and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The extent of the reduction of MTT was determined by measurement of the absorbance at 550 nm.

Flow Cytometry Analysis for Apoptosis. PC12 cells (2×10^6/mL) were seeded in 6-well plates and cultured in serum-free RPMI-1640 medium supplemented with indicated reagent or DMSO vehicle (final concentration of 0.1%) for 48 h. After treatment, PC12 cells were washed twice with PBS and fixed with ice-cold 70% ethanol for 2 h. The cells were then centrifuged at 1,500*g* for 5 min and washed with PBS twice. Cells were stained with 1 mL of PI-staining solution (PBS containing 20 μg/mL of propidium iodide, 200 μg/mL of RNase, and 0.1% Triton X-100) and incubated in the dark at 4 °C for 30 min. The cells were washed twice and suspended in PBS containing 0.1% sodium azide solution before flow cytometry analysis (FACSCalibur, BD Biosciences, San Jose, CA, USA). In the DNA histogram, the amplitude of the sub-G1 DNA peak represents the number of apoptotic cells.

Reverse Transcription Quantitative PCR (RT-Q-PCR) Analysis of GAP-43 and HO-1 mRNA Expression. PC12 cells (2×10^6/mL) were seeded on poly-L-lysine-coated 6-well plates in normal medium for 24 h. The cells were then shifted to low serum (1% HS and 0.5% FBS) or serum-free RPMI medium as indicated for 24 h prior to exposure to the indicated reagent or DMSO vehicle (final concentration of 0.1%) for the indicated period. Total cellular RNA was prepared using RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK). Reverse transcription of 2 μg of RNA was performed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed with 2 μL of cDNA obtained as described above in 25 μL containing 200 nM primers [HO-1, 5′-GCC-TGCTAGCCTGGTTCAAG-3′ (forward) and 5′-AGCGGTGTCTGG-GATGAACTA-3′ (reverse); GAP-43, 5′-CTAAGGAAAGTGCCCGAC-AG-3′ (forward) and 5′-GCAGGAGAGACAGGGTTCAG-3′ (reverse); β-actin, 5′-CCTCTGAACCCTAAGGCCAA-3′ (forward) and 5′-AGCC-TGGATGGCTACGTACA-3′ (reverse)] and Power SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an ABI Prism 7300 Real-Time PCR system. PCR conditions were as follows: 94 °C for 4 min, 40 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The $\Delta\Delta C_t$ method was used for data analysis of GAP-43 and HO-1 mRNA expression estimated in triplicate samples and normalized to β-actin expression levels.

Western Blotting Analysis of GAP-43, HO-1, and ERK Proteins. PC12 cells (2×10^6/mL) were seeded on poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h, then shifted to low serum (1% HS and 0.5% FBS) or serum-free medium as indicated for 24 h prior to exposure to the indicated reagent or vehicle (0.1% DMSO) for the indicated period. Cells were washed with PBS, scraped in ice cold RIPA buffer (Thermo Fisher Scientific, Inc., Rockford, IL), and incubated on ice for 15 min. The cellular debris was removed by centrifugation (8,000*g* for 15 min) at 4 °C, and the cell lysate was carefully transferred to the microcentrifuge tube. The protein concentration was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

Cell lysate (30 μg) was separated on 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto Hybond ECL nitrocellulose (GE Healthcare) at 20 V overnight at 4 °C. The membranes were blocked at 4 °C in PBST blocking buffer (1% nonfat dried milk in PBS containing 0.1% Tween-20) for 8 h. Blots were incubated with the appropriate antibodies overnight at 4 °C: anti-GAP-43 (1:1000) (Millipore, Billerica, MA, USA), anti-ERK1/2 (1:1000), antiphospho-ERK1/2 (1:1000) (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-HO-1 (1:1000) (Stressgen Biotech Corp., Victoria, BC, Canada), and anti-β-actin (1:8000) (Sigma-Aldrich). After three washes with PBST, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000) for 1 h. The blots were washed again and the proteins of interest detected by Amersham ECL Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer's instructions. Then, the chemiluminescence signal was visualized with X-ray film.

Analysis for the ARE Binding Activity of Nuclear Factor E2-Related Factor 2 (Nrf2). PC12 cells (2×10^6/mL) were seeded on poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h then replaced with serum-free RPMI-1640 medium for 24 h prior to exposure to the indicated reagent or vehicle (0.1% DMSO) for the indicated period. Nuclear extracts were harvested using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce Endogen, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration of the nuclear extract was measured using the Bio-Rad protein assay reagent, and the

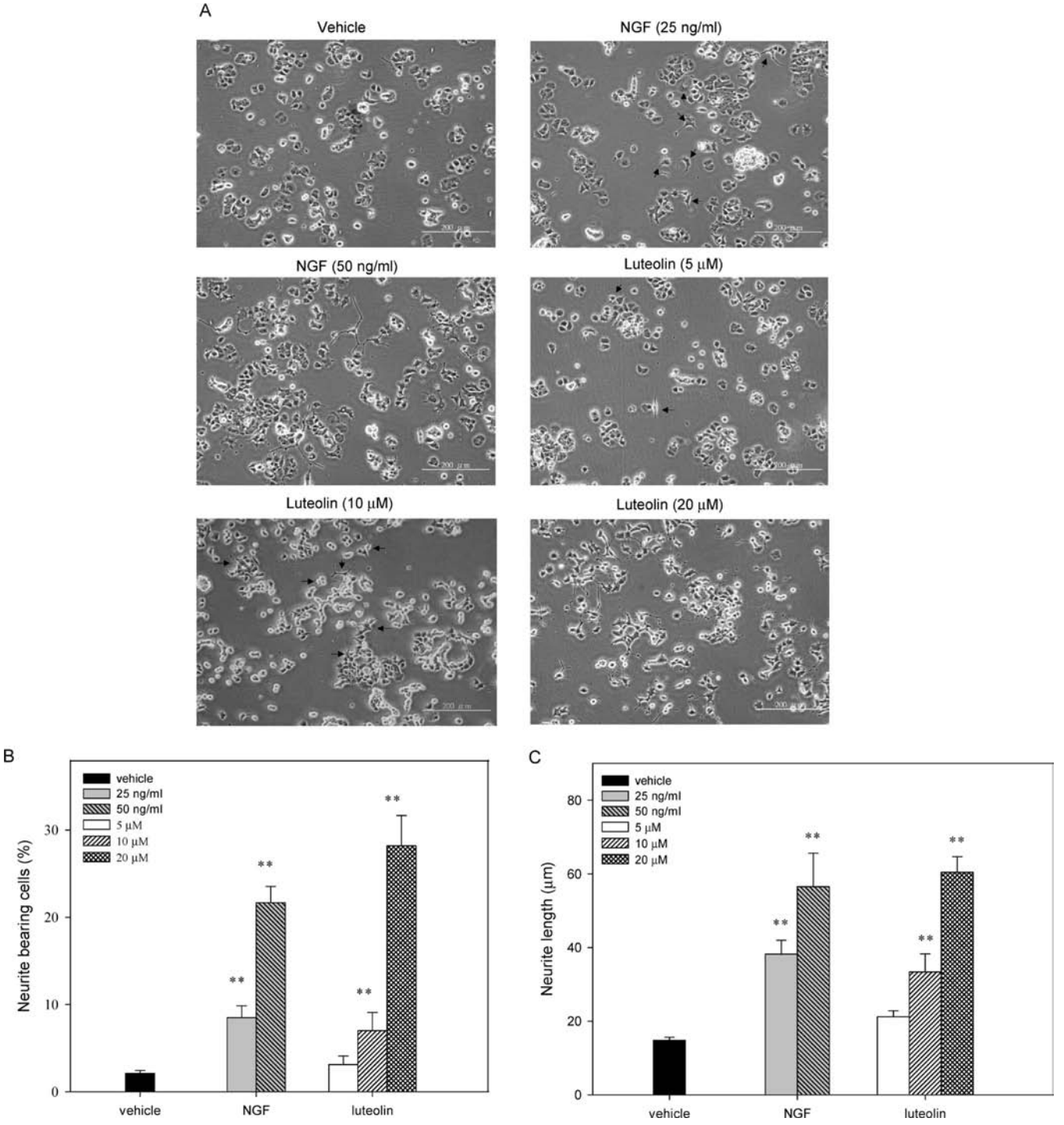

Figure 2. Effect of luteolin on neurite outgrowth in PC12 cells. (**A**) PC12 cells were seeded on poly-L-lysine-coated 6-well plates in normal medium for 24 h and then shifted to low serum medium (1% HS and 0.5% FBS) for 24 h prior to exposure to vehicle (0.1% DMSO), NGF (25 and 50 ng/mL), or luteolin (5, 10, and 20 μM) for an additional 72 h. Cell morphology was observed using phase-contrast microscopy. Arrowheads indicate the neurite bearing cells in NGF (25 ng/mL)- and luteolin (5, 10 μM)-treated groups. (**B**) Neurite bearing cells were counted under phase-contrast microscopy in five random fields, and data represent the mean $\pm$ SEM from three independent experiments. (**C**) The maximal neurite length for each of the differentiated cells was analyzed by Image J software. Data represent the mean $\pm$ SEM from three independent experiments. $^{**}p < 0.01$ represents significant differences compared with that of vehicle-treated cells.

amount of Nrf2 available in the nucleus to bind to ARE (antioxidant response element) was determined by a TransAM Nrf2 Transcription Factor ELISA kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, nuclear extracts were added to wells containing the immobilized consensus ARE oligonucleotide. A primary antibody against Nrf2 was added to each well. Then a secondary antibody conjugated to horseradish peroxidase that binds to the primary (Nrf2) antibody was added to each well. The signal was detected at 450 nm, and Nrf2-ARE binding was reported as optical density units at 450 nm.

Statistical Analysis. All experiments were repeated at least three times. The results were analyzed by Student's unpaired *t*-test, and a *p* value of < 0.05 was taken to be significant.

RESULTS

Luteolin Induces Neurite Outgrowth of PC12 Cells. The PC12 cell line is widely used as a cellular model for studies of neuronal differentiation (*5*). To investigate the neuritogenic action, adherent

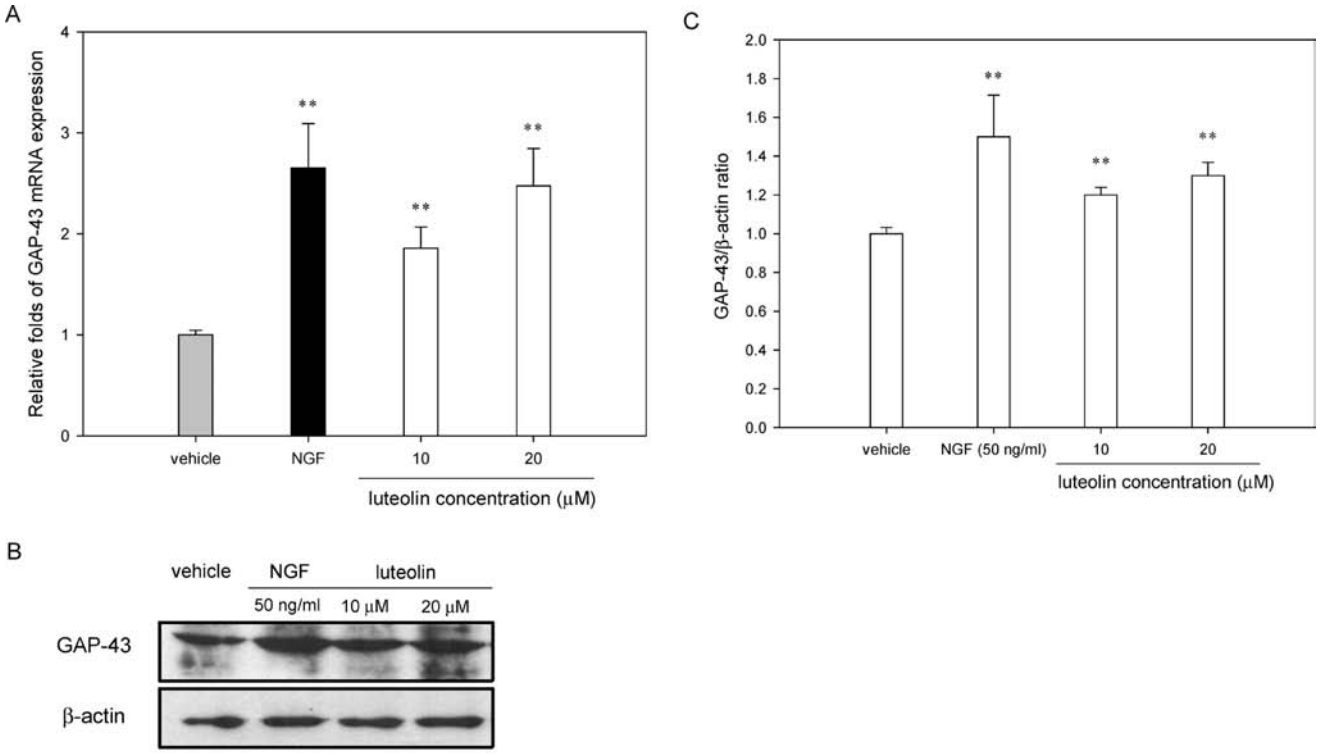

Figure 3. Effect of luteolin on GAP-43 mRNA and protein expression in PC12 cells. PC12 cells were seeded on poly-L-lysine-coated 6-well plates in normal medium for 24 h and then shifted to low serum medium (1% HS and 0.5% FBS) for 24 h prior to exposure to the indicated agent. (**A**) Adherent PC12 cells were treated with NGF (50 ng/mL) for 24 h or luteolin (10 and 20 μM) for 72 h. RNA was then prepared, and the GAP-43 mRNA level was detected by RT-Q-PCR as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. (**B**) Adherent PC12 cells were treated with vehicle (0.1% DMSO), NGF (50 ng/mL), or luteolin (10 and 20 μM) for 72 h. GAP-43 protein expression was detected by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated three times, and a representative blot was shown. (**C**) Normalized intensity of GAP-43 versus β-actin is presented as the mean ± SEM of three independent experiments. $^{**}p < 0.01$ represents significant differences compared with that of vehicle-treated cells.

PC12 cells were maintained in low serum medium (1% HS and 0.5% FBS) and treated with vehicle (0.1% DMSO), nerve growth factor (NGF, 25 and 50 ng/mL), or luteolin (5, 10, and 20 μM) for 72 h before observation and counting neurite bearing cells under microscopy (**Figure 2A**). As shown in **Figure 2B** and **C**, 10 and 20 μM luteolin markedly increased both the percentage of neurite bearing cells (7.0 ± 2.1% and 28.2 ± 3.5%, respectively) and the maximal neurite length in those cells (33.3 ± 5.0 μm and 60.4 ± 4.2 μm, respectively) as compared with those in the vehicle (2.1 ± 0.3% in numbers and 14.8 ± 0.9 μm in length) ($p < 0.01$). The activity was comparable to those of the positive control, NGF.

Luteolin Up-Regulates the Expression of the Neuronal Differentiation Biomarker of PC12 Cells. Growth-associated protein-43 (GAP-43) is a neuron-specific protein which exhibits elevated synthesis and axonal fast-transport during nerve regeneration and also serves as a differentiation biomarker of PC12 cells (*18*). Adherent PC12 cells were maintained in medium containing 1% HS and 0.5% FBS for 24 h, then treated with vehicle (0.1% DMSO), NGF (50 ng/mL), or the indicated concentration of luteolin for the indicated period. GAP-43 mRNA and protein expression were analyzed by reverse transcription quantitative PCR (RT-Q-PCR) and Western blot, respectively, and the results are shown in **Figure 3**. It has been reported that NGF stimulated the maximal expression of GAP-43 mRNA after 24 h of exposure in PC12 cells (*18*). **Figure 3A** shows the exposure of PC12 cells to NGF (50 ng/mL) for 24 h, GAP-43 mRNA level increased by 2.6-fold as compared with that in the vehicle control. Cells treated with 10 and 20 μM luteolin for 72 h also caused 1.9- and 2.5-fold increases in the GAP-43 transcript, respectively. Western blot analysis further revealed that the protein expression of GAP-43 was induced significantly by NGF and luteolin after 72 h of treatment (**Figure 3B**). GAP-43 protein levels were elevated 1.5- and 1.3-fold in response to NGF (50 ng/mL) and luteolin (20 μM), respectively (**Figure 3C**). These results indicate that luteolin mimics a neuritogenic action of NGF in stimulating PC12 cell differentiation dose-dependently.

Luteolin Protects the PC12 Cells from Serum Withdrawal-Induced Cell Death. Nondifferentiated, proliferating PC12 cells undergo cell death upon withdrawal of the neurotrophic factor or serum (*4*). To investigate whether luteolin possesses a neuroprotective effect against serum deprivation-induced cell death, MTT assay was performed for the determination of cell viability. Values were expressed as the percentage of control, with the value obtained for normal serum cultured cells taken before shifting to serum-free medium as 100%. NGF served as a positive control for cell survival and growth. In **Figure 4A**, PC12 cells shifted to serum-free medium supplemented with 0.1% DMSO (vehicle) for 48 h showed a significant decrease in cell viability (43.1 ± 1.9%). The cell viability increased to 63.4 ± 1.2% ($p < 0.05$), 78.9 ± 0.4% ($p < 0.01$), and 84.7 ± 5.3% ($p < 0.01$) for those supplemented with 5, 10, and 20 μM of luteolin, respectively.

We further analyzed the cell cycle status of PC12 cells by staining the cellular DNA with propidium iodide (PI) (**Figure 4B**). When PC12 cells were cultured in serum-free medium supplemented with 0.1% DMSO (vehicle), they entered into apoptosis, which was evident after 48 h (30.3 ± 2.6% of cells in sub-G1) as

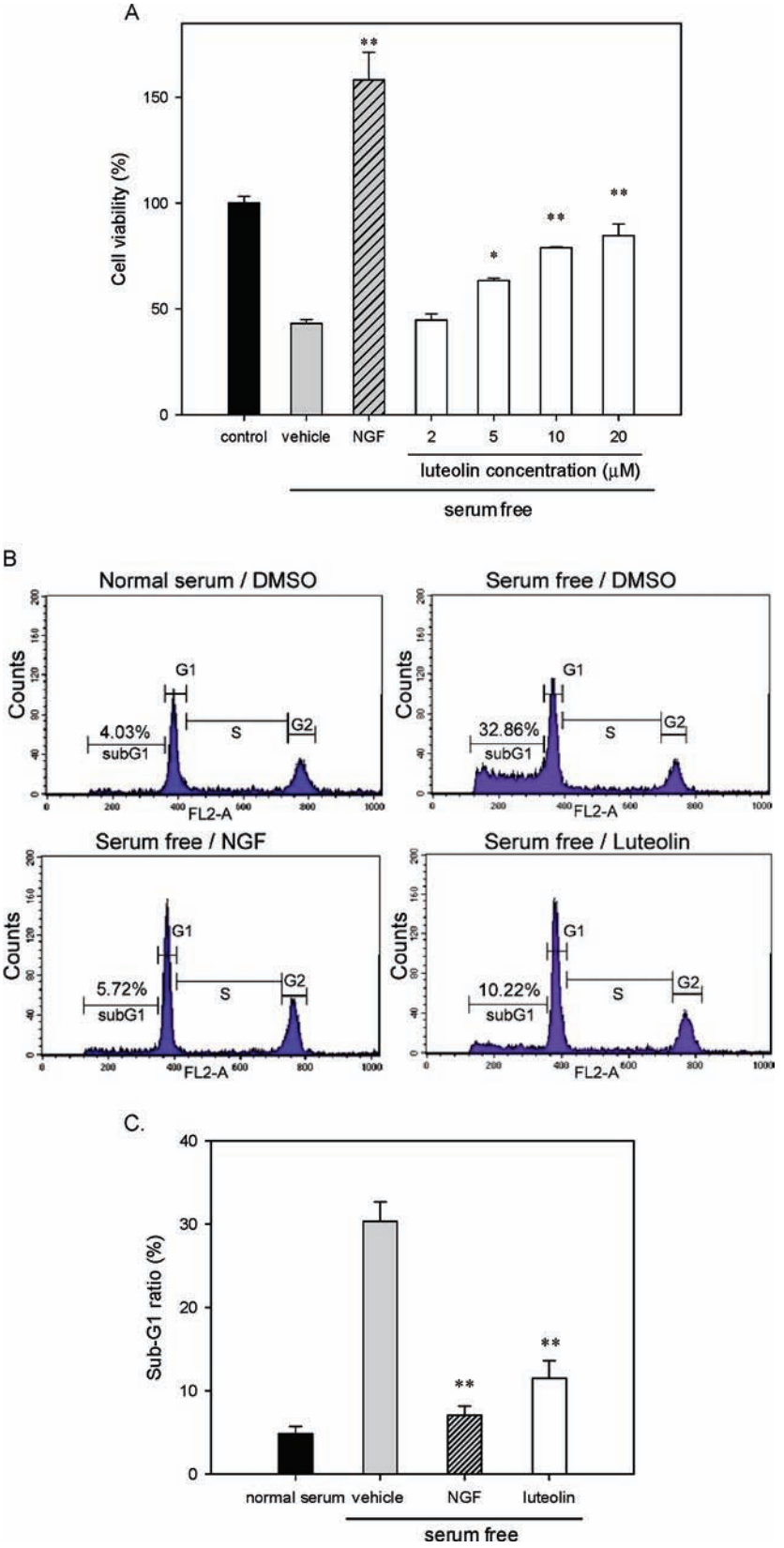

Figure 4. Effect of luteolin in preventing PC12 cells from serum deprivation-induced apoptosis. PC12 cells were cultured in serum-free medium containing the indicated agent or vehicle (0.1% DMSO) for 48 h. (**A**) Cell viability was measured by MTT assay. Values are expressed as percentage of control. (**B**) Cell cycle analysis of PC12 cells cultured in serum-free medium supplemented with indicated agent or vehicle (0.1% DMSO) for 48 h. After fixation with ethanol for 2 h, cells were stained with PI (20 μg/mL) and detected by flow cytometry. Apoptotic ratio of PC12 cells was determined by a sub-G1 peak. (**C**) Summary of the apoptotic ratio of different treatments. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences compared with those of the vehicle-treated cells.

compared with those cultured in normal serum conditions (4.8 ± 0.9% of cells in sub-G1). The ratio of apoptotic cells was reduced to 7.1 ± 1.5% and 11.5 ± 2.1% ($p < 0.01$) by the addition of NGF (50 ng/mL) and luteolin (20 μM), respectively (**Figure 4C**).

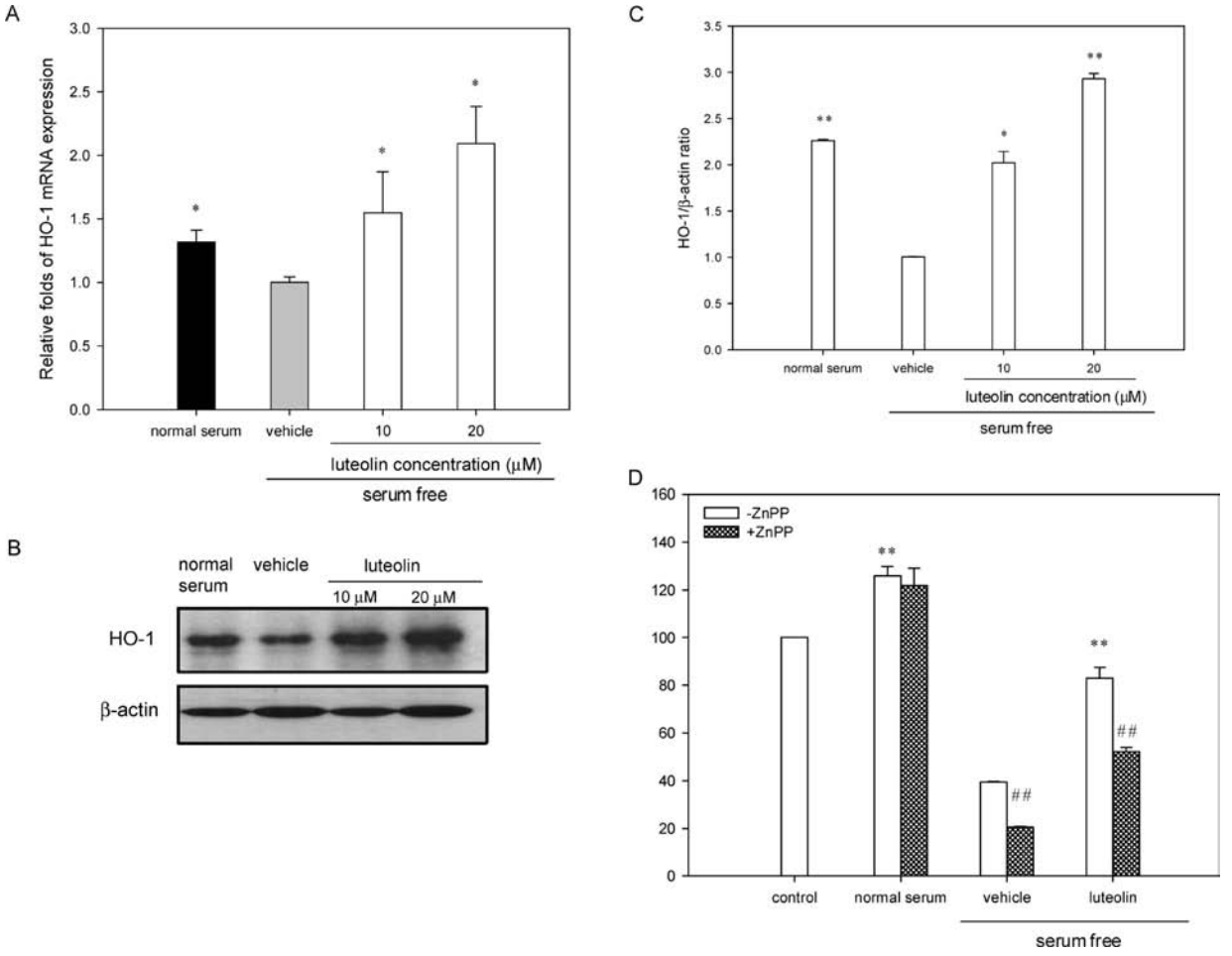

Figure 5. Effect of luteolin on HO-1 mRNA and protein expression in PC12 cells. PC12 cells were seeded on poly-L-lysine-coated 6-well plates in normal medium for 24 h and then shifted to serum-free medium for 24 h prior to exposure to the indicated agent. (**A**) Adherent PC12 cells were treated with vehicle (0.1% DMSO) or luteolin (10 and 20 μM) for 6 h. RNA was then prepared, and HO-1 mRNA expression was analyzed by RT-Q-PCR as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. (**B**) Adherent PC12 cells were treated with vehicle (0.1% DMSO) or luteolin (10 and 20 μM) for 9 h, and HO-1 protein was detected using Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot was shown. (**C**) Normalized intensity of HO-1 versus β-actin is presented as the mean ± SEM of three independent experiments. (**D**) PC12 cells were treated with vehicle (0.1% DMSO) or luteolin (20 μM) in the presence or absence of Znpp (1 μM) in normal or serum free medium for 48 h. Cell viability was measured by MTT assay. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences compared with those of the vehicle-treated cells. ##$p < 0.01$ represents significant differences compared with those of the respective Znpp-untreated group.

Similar to the MTT result shown above, flow cytometry data indicate that luteolin and NGF attenuated serum deprivation-induced PC12 cell apoptosis.

Luteolin Induces HO-1 Expression in PC12 Cells. It has been reported that up-regulation of the expression and activation of heme oxygenase-1 (HO-1) is responsible for protecting cells from oxidative stress-induced cell injury (*19*). As a result, we further investigated whether luteolin could alter the expression of HO-1 so as to protect PC12 cells from serum deprivation-induced oxidative damage. Adherent PC12 cells were maintained in serum-free medium for 24 h, then treated with vehicle (0.1% DMSO) or the indicated concentration of luteolin for another 6 h, and HO-1 mRNA expression analyzed by RT-Q-PCR. In accordance with the previous report (*20*), cells cultured in normal serum medium demonstrated a slightly increased level of HO-1 mRNA (1.3 ± 0.1-fold; $p < 0.05$) compared with that of cells grown in vehicle containing serum-free medium normalized to β-actin expression (**Figure 5A**). Luteolin (10 and 20 μM) increases HO-1 mRNA expression as compared with the vehicle control (1.6 ± 0.3- and 2.1 ± 0.3-fold; $p < 0.05$).

The HO-1 protein expression after the treatment of luteolin was further determined by Western blotting analysis. The immunoblot analysis showed that HO-1 levels increased slightly after 6 h of exposure of cells to luteolin (data not shown) and dramatically expressed after 9 h of treatment of PC12 cells (**Figure 5B**). Quantification of the Western blot data revealed that cells cultured in serum-free medium supplemented with 10 and 20 μM luteolin for 9 h caused 2.0- and 2.9-fold increases in HO-1 protein expression, as compared with that in the vehicle control (**Figure 5C**). This result indicates that luteolin could induce HO-1 expression dose-dependently in PC12 cells under serum-free conditions.

To further investigate whether luteolin-induced HO-1 activity could confer cytoprotection, PC12 cells were treated with luteolin in the absence or presence of the HO-1 enzyme inhibitor zinc protoporphyrin (Znpp) for 48 h in serum-free medium. As shown in **Figure 5D**, the addition of Znpp (1 μM), the competitive inhibitor of HO-1 enzyme activity, did not exert cytotoxicity in cells cultured in normal serum conditions. However, when PC12 cells were cultured in serum-free medium, Znpp (1 μM) caused

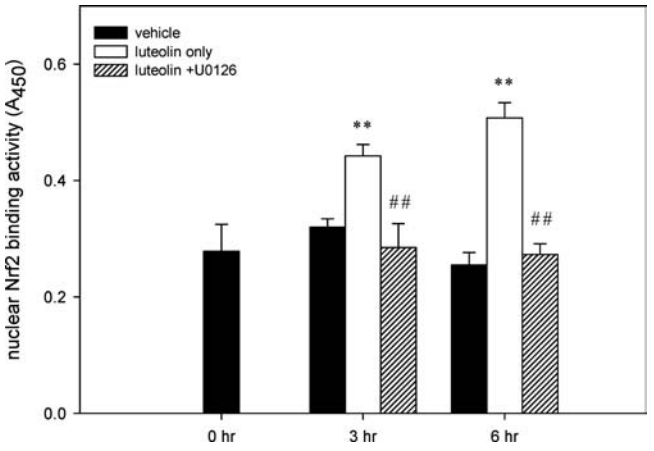

Figure 6. Effect of luteolin on ARE binding activity of nuclear Nrf2. PC12 cells were seeded on poly-L-lysine-coated 100 mm dishes in normal medium and shifted to serum-free medium for 24 h prior to exposure to vehicle (0.1% DMSO) or luteolin (20 μM) in the presence or absence of U0126 (10 μM) for 3 or 6 h. Nuclear extracts were isolated, and the binding activity of nuclear Nrf2 on the antioxidant response element (ARE) was analyzed as described in Materials and Methods. Data represent the mean $\pm$ SEM of three independent experiments. **$p < 0.01$ represents significant differences compared with that of the vehicle. ##$p < 0.01$ represents significant differences compared with that of the respective U0126-untreated group.

significant cytotoxicity as compared with the Znpp-untreated group (about 19% decrease in cell viability, $p < 0.01$). Consistent with **Figure 4A**, treatment of PC12 cells with luteolin (20 μM) in serum-free conditions, markedly protected them from serum-deprivation induced cytotoxicity ($p < 0.01$); while the addition of Znpp (1 μM) reversed the protection action and led to a 33% reduction in cell viability as compared with the Znpp-free group ($p < 0.01$). This result suggests that HO-1 activity contributes, at least in part, to the cytoprotective effect of luteolin when serum is withdrawn.

Luteolin Activates Nuclear Nrf2-ARE Binding. The antioxidant response element (ARE) is a cis-acting enhancer sequence found in the promoter region of many genes encoding antioxidant and phase II detoxification enzymes/proteins. The major ARE-binding transcription factor, NF-E2-related factor 2 (Nrf2), induces mRNA expression of the ARE-driven HO-1 gene (*21*). To examine whether luteolin can enhance ARE-binding activity of nuclear Nrf2, PC12 cells in serum-free conditions were incubated with the vehicle (0.1% DMSO) or luteolin (20 μM) for the indicated period, and the ARE-binding activity of the nuclear extract was analyzed using the ELISA-based TransAM Nrf2 kit as described in Materials and Methods. **Figure 6** shows that treatment of PC12 cells with luteolin in serum-free conditions for 3 and 6 h caused 1.6- and 1.8-fold increases in the amount of nuclear Nrf2 bound to ARE as compared with that in the vehicle ($p < 0.01$).

Luteolin Promotes ERK Phosphorylation in PC12 Cells. It has been well revealed that extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation may activate various protein signaling cascades and downstream transcription factors, thereby influencing a large variety of cellular processes, such as cell differentiation, survival, and cell-cycle regulation (*22*). To investigate whether luteolin can activate ERK signaling pathways, adherent PC12 cells maintained under serum-free conditions for 24 h were treated with vehicle (0.1% DMSO) or luteolin (20 μM) for the indicated period, and the cell lysate was immunoblotted with ERK1/2 and phospho-ERK1/2 specific antibodies

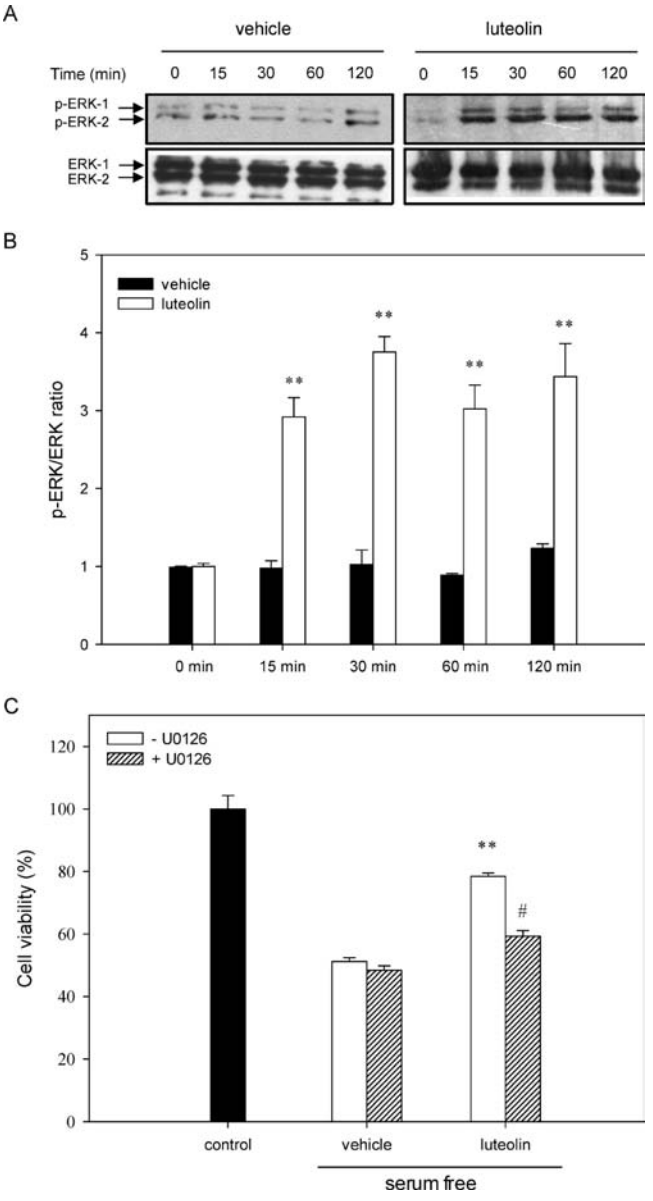

Figure 7. Involvement of ERK activation in luteolin-induced cytoprotective action in PC12 cells. PC12 cells were seeded on poly-L-lysine-coated 100 mm dishes in normal medium and shifted to serum-free medium for 24 h prior to exposure to the indicated agent. (**A**) Adherent PC12 cells were treated with vehicle (0.1% DMSO) or luteolin (20 μM) for 0, 15, 30, 60, or 120 min. Phospho-ERK1/2 and ERK1/2 were analyzed by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot was shown. (**B**) Normalized intensity of p-ERK1/2 versus ERK1/2 is presented as the mean $\pm$ SEM of three independent experiments. (**C**) Adherent PC12 cells were pretreated with U0126 (10 μM) for 30 min, then incubated with luteolin (20 μM) for an additional 48 h. Cell viability was measured by MTT assay. Data represent the mean $\pm$ SEM of three independent experiments. **$p < 0.01$ represents significant differences compared with that of vehicle-treated cells. #$p < 0.05$ represents significant differences compared with that of the respective U0126-untreated group.

as described in Materials and Methods. As shown in **Figure 7A** and **B**, treatment of PC12 cells with luteolin caused approximately 3-fold ERK1/2 phosphorylation after 15 min and remained elevated up to 120 min as compared with that in the vehicle group.

To determine whether ERK1/2 activation relates to luteolin-mediated neuroprotection, PC12 cells were pretreated with

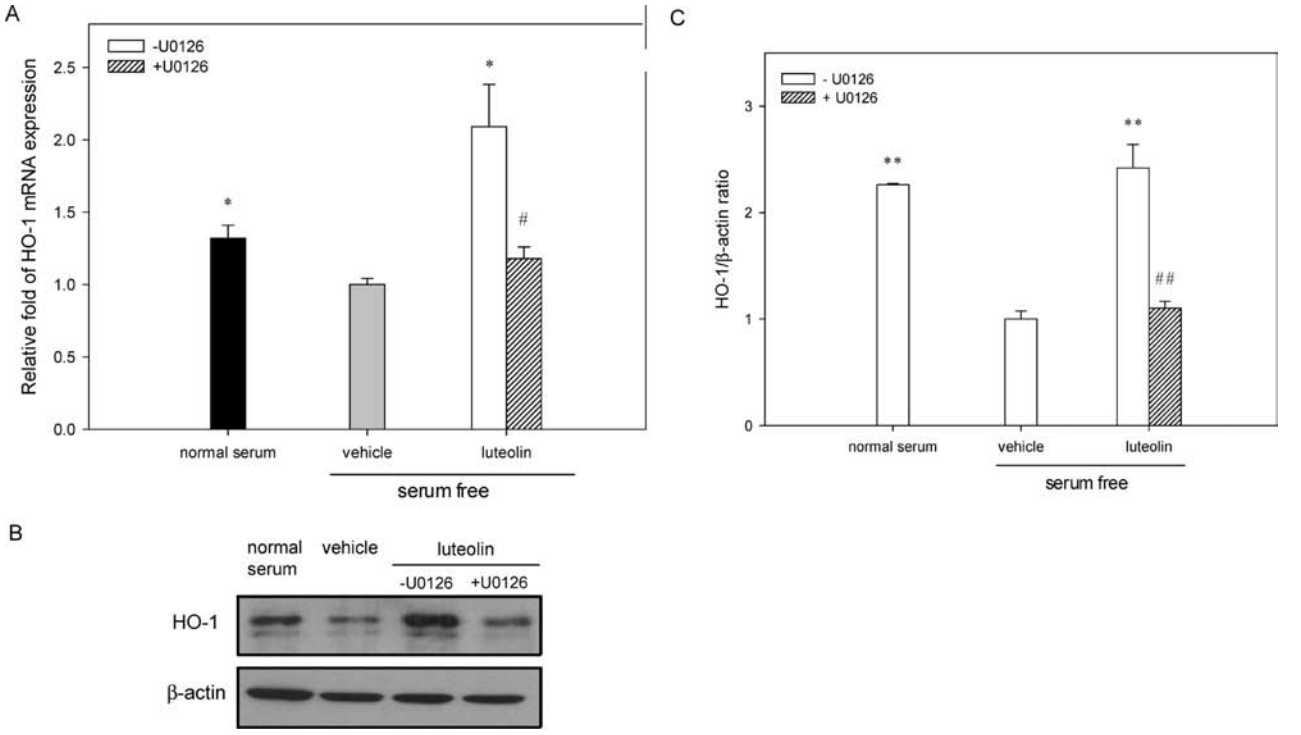

Figure 8. Effect of ERK inhibitor on the induction of HO-1 by luteolin. PC12 cells were seeded on poly-L-lysine-coated 100 mm dishes in normal medium and shifted to serum-free medium for 24 h prior to exposure to U0126 (10 μM) for 30 min. (**A**) Adherent PC12 cells were then incubated with vehicle (0.1% DMSO) or luteolin (20 μM) for 6 h, and HO-1 mRNA expression was determined by RT-Q-PCR as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. (**B**) Adherent PC12 cells were then incubated with vehicle (0.1% DMSO) or luteolin (20 μM) for 9 h, and HO-1 protein was detected using Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot is shown. (**C**) Normalized intensity of HO-1 versus β-actin is presented as the mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences compared with those of the vehicle-treated cells in serum free conditions. #$p < 0.05$ and ##$p < 0.01$ represent significant differences compared with those of the respective U0126-untreated group.

U0126 (10 μM), a specific inhibitor of ERK1/2 kinase, for 30 min and then incubated with 20 μM luteolin for 48 h under serum-free medium before analyzing cell viability. As shown in **Figure 7C**, the concentration of U0126 (10 μM) did not exert significant cytotoxicity for PC12 cells cultured in serum-free conditions. However, the cell viability of luteolin-treated PC12 cells was significantly reduced from 78.4 ± 1.1% to 59.3 ± 1.8% ($p < 0.05$). This result indicates that ERK activation plays an important role in luteolin-mediated cytoprotection against serum-deprived oxidative damage.

To further analyze whether ERK is involved in channeling the stimulus for Nrf2 activation and HO-1 upregulation, PC12 cells maintained under serum withdrawal conditions for 24 h were first incubated with U0126 (10 μM) for 30 min and then treated with luteolin (20 μM) for 3 and 6 h before the determination of Nrf2 DNA binding activity. As shown in **Figure 6**, luteolin-mediated nuclear Nrf2 activation was completely abolished by U0126 to the level of the vehicle ($p < 0.01$).

The effect of U0126 on luteolin-induced HO-1 mRNA and protein expression was also evident. As expected, luteolin (20 μM) significantly activated HO-1 mRNA and protein expression after 6 and 9 h of incubation, respectively (**Figure 8A−C**). The addition of U0126 (10 μM) reversed the induction to the level of the vehicle. These results indicate that ERK activation is essential for luteolin-induced Nrf2 activation and subsequent HO-1 upregulation.

Involvement of ERK and Protein Kinase C Signaling Pathways in Luteolin-Induced PC12 Differentiation. It has been demonstrated that ERK and protein kinase C (PKC) activation are involved in NGF-induced neurite outgrowth in PC12 cells (*23*). To further investigate whether luteolin-induced neurite outgrowth is also dependent on the activation of ERK and PKC signaling, adherent PC12 cells were maintained in low serum medium (1% HS and 0.5% FBS) and treated with U0126 (10 μM) or bisindolylmaleimide I (BIM, 1.25 μM and 2.5 μM) for 30 min prior to the addition of luteolin (10 and 20 μM) for 72 h. **Figure 9A** demonstrated that inhibition of ERK activation significantly attenuated the neurite bearing cells from the original 8.3 ± 0.7% to 5.4 ± 0.8% and 26.5 ± 3.2% to 7.8 ± 1.6%, for those treated with 10 and 20 μM luteolin, respectively. However, exposure of cells to BIM (1.25 and 2.5 μM), a highly selective, cell-permeable, and reversible PKC inhibitor, caused a less dramatic reduction in the percentage of neurite bearing cells in 20 μM luteolin-treated cells (17.9 ± 2.0% and 15.2 ± 1.5%, respectively) (**Figure 9B**). This result indicates both ERK and PKC signaling pathways are involved in luteolin-mediated PC12 cell differentiation.

DISCUSSION

There is mounting evidence supporting the fact that oxidative stress is implicated as a major cause of cellular injuries in a variety of human diseases including neurodegenerative disorders. One of the feasible ways to prevent oxidative stress-mediated cellular injuries is to augment the oxidative defense capacity through the intake of antioxidants. Recently, attention has been focused on nonvitamin antioxidants, such as polyphenolic compounds, that are able to scavenge free radicals and protect cells from oxidative damage. For example, the neuroprotective action and molecular mechanism of (−)-epigallocatechin-3-gallate (EGCG) have been well-documented as the outcome of antioxidant, iron chelating, and neuritogenic properties (*24*). Luteolin is a well known strong

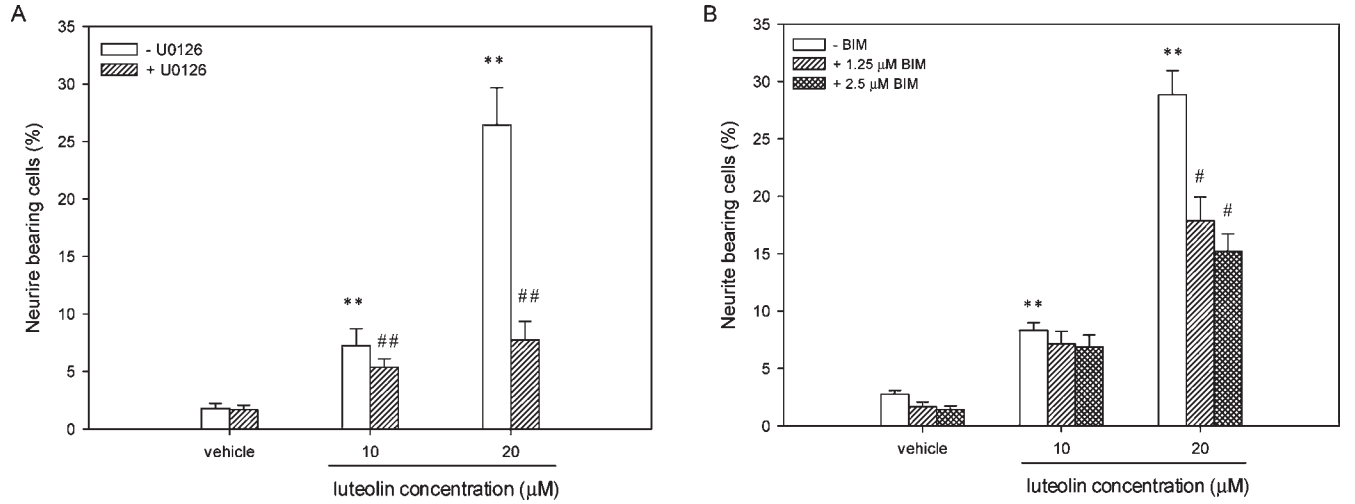

Figure 9. Effects of ERK and PKC inhibitors on luteolin-induced neurite outgrowth. PC12 cells were seeded on poly-L-lysine-coated 6-well plates in normal medium for 24 h. Cells were then shifted to low serum medium (1% HS and 0.5% FBS) for 24 h and then were pretreated with (**A**) U0126 (10 μM) or (**B**) bisindolylmaleimide I (BIM, 1.25 μM and 2.5 μM) for 30 min. Cells were then exposed to vehicle (0.1% DMSO) or luteolin (10 μM and 20 μM) for 72 h. Neurite bearing cells were counted under phase-contrast microscopy as described in Materials and Methods. Data represent the mean ± SEM from three independent experiments. $^{**}p < 0.01$ represents significant differences compared with that of the vehicle-treated cells. $\#p < 0.05$ and $\#\#p < 0.01$ represent significant differences compared with those of the respective U0126- or BIM-untreated group.

ROS scavenger and serves as an antioxidant, anti-inflammatory, and anticancer agent (*14*). It has also been indicated as a promising orally available, blood−brain barrier permeable compound for the therapy of neurodegenerative disorders (*17*). Together with its moderate ferrous ion chelating activity in vitro (data not shown), luteolin is likely to exert an effect similar to that of EGCG in neuroprotection. It may activate a hypoxia signal transduction pathway that culminates in the stabilization of hypoxia inducible factor (HIF)-1 and increased transcription of neurotrophic genes mediating compensatory neuronal survival and differentiation (*24*).

In the current study, we first demonstrated that luteolin as low as 5 μM was effective in defending PC12 cells against serum deprivation-induced oxidative stress by using the MTT assay (**Figure 4A**) and cell cycle analysis (**Figure 4B**). The protective effect of luteolin seems compatible with that of NGF. We then took one step further to explore the molecular mechanisms underlying the protective action of luteolin, with focus on the up-regulation of the HO-1 and Nrf2-ARE pathway. HO-1, a phase II cytoprotective enzyme, is a ubiquitous and redox-sensitive inducible stress protein (*25*) that is transcriptionally regulated by a variety of stimuli. It has been widely accepted that increasing HO-1 expression represents an adaptive response that confers resistance to oxidative injury (*20*).

In this research, we found that treatment of PC12 cells with luteolin induced both HO-1 mRNA and protein expression (**Figure 5A−C**). HO-1 activity was further proved to be involved in PC12 cell survival from oxidative stress because the addition of Znpp, the competitive inhibitor, substantially decreased cell viability (**Figure 5D**). The addition of Znpp (1 μM) exerts no cytotoxicity in PC12 cells cultured in normal serum medium. This data is consistent with our previous study (*11*). However, in serum-free conditions, Znpp caused reductions in cell viability for vehicle- and luteolin-treated cells by approximately 19% and 33%, respectively (**Figure 5D**). This result suggests that HO-1 activity is involved in PC12 cell survival from oxidative stress.

Several studies have independently demonstrated that HO-1 up-regulation was mediated by activation, nuclear translocation, and selective ARE binding of Nrf2 (*20*, *26*). It has been reported that primary neuronal cultures treated with chemical activators of the Nrf2-ARE pathway displayed significantly greater resistance to oxidative stress-induced neurotoxicity (*27*). Therefore, it was of particular interest to determine whether luteolin could activate Nrf2 in association with HO-1 up-regulation in PC12 cells. As illustrated in **Figure 6**, treatment of luteolin causes elevated Nrf2 activation, which correlates with a marked increase in HO-1 expression (**Figure 5**). These results support the fact that luteolin protects PC12 cells from serum withdrawal-induced oxidative stress through Nrf2-mediated transcriptional activation of HO-1.

Most studies on the regulation of phase II gene expression have focused on the role of MAPK pathways (*28*). It is known that MAPKs are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAPK kinases. In this work, we provide evidence that luteolin treatment increased a sustained rise in the phosphorylation of ERKs (**Figure 7A** and **B**). The use of a specific inhibitor for ERK1/2 upstream kinase MEK1/2, U0126, significantly attenuated luteolin-induced Nrf2 binding to ARE (**Figure 6**) and the expression of HO-1 mRNA and protein (**Figure 8A−C**). These data confirm the involvement of ERK signaling in luteolin-mediated HO-1 induction.

It has been known that a neurotrophic factor, such as NGF, is essential not only for PC12 cell viability in serum-deprived conditions but also for cell differentiation (*6*). Herein, we found that luteolin dose-dependently stimulated neurite outgrowth in both maximal neurite length and percentage of neruite bearing cells (**Figure 2A−C**). The neuronal differentiation-promoting activity of luteolin is comparable to that of NGF in PC12 cells. It is known that the growing neurons express high levels of GAP-43 (*18*) and that the up-regulation of GAP-43 mRNA and protein is associated with the differentiation of PC12 cells into the neuronal phenotype (*23*). Our data show that after exposure to NGF (50 ng/mL) for 24 h, PC12 cells exhibited morphological changes and neurite formation (data not shown) along with elevated expression of GAP-43 mRNA (**Figure 3A**). However, in luteolin-stimulated PC12 cells, neurite bearing cells were only found after 48−72 h of treatment, together with the increased mRNA and protein levels of GAP-43 (**Figure 3A** and **B**).

It has been shown that NGF promotes survival and enhances the differentiation of nerve cells via the activation of the ERK pathway (*29*). It is also reported that PKC activity regulates the

level of the GAP-43 mRNA in PC12 cells (*30*). Herein, we found that the addition of U0126 caused more significant decrease in the percentage of neurite bearing cells than the addition of BIM, a PKC inhibitor, in luteolin-treated cells (**Figure 9**). This result shows the involvement of both ERK and PKC signaling pathways in PC12 differentiation, and the former seems more critical for luteolin-induced neurite outgrowth. In summary, the current findings demonstrate that the ERK signaling pathway plays an essential role in luteolin-mediated neurotrophic and neuroprotective activity in PC12 cells.

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