Protection of Cultured Cortical Neurons by Luteolin against Oxidative Damage through Inhibition of Apoptosis and Induction of Heme Oxygenase-1

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Received July 15, 2016; accepted December 19, 2016

Luteolin, one of the most common flavonoids present in many types of natural products, possesses diverse biological properties including anti-oxidant activity. In this study, we investigated neuroprotective effect of luteolin and its underlying signaling pathways using primary cultured rat cortical cells. Luteolin was demonstrated to attenuate 

H2O2- or xanthine/xanthine oxidase-induced oxidative damage and generation of intracellular reactive oxygen species (ROS). It enhanced the phosphorylation of Bad at Ser112 and attenuated H2O2-induced activation of caspase 3, indicating anti-apoptotic action. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay confirmed this finding, showing inhibition of 

H2O2-induced DNA fragmentation. We also found that luteolin significantly up-regulated the expression of anti-oxidant enzyme heme oxygenase (HO)-1. Treatment with tin protoporphyrin IX, a selective HO-1 inhibitor, abolished neuroprotective and anti-apoptotic effects of luteolin, suggesting a critical role of HO-1 up-regulation. It was also shown to increase the phosphorylation of mitogen-activated protein kinase (MAPKs) such as extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinases (JNK) and Akt. Treatment of the cells with specific inhibitors including SB203580, SP600125, and LY294002 suppressed the luteolin-induced HO-1 expression, suggesting the involvement of p38 MAPK, JNK, and Akt in HO-1 induction. In contrast, HO-1 expression was not reduced by U0126, implying that ERK may not be directly involved in HO-1 induction. These results indicate that luteolin exhibits neuroprotective effect through the inhibition of ROS and apoptotic cell death. Furthermore, up-regulation of HO-1 expression via p38 MAPK, JNK and Akt may contribute, at least in part, to luteolin-mediated neuroprotection. Based on these findings, luteolin may serve as a potential intervention for neurodegenerative diseases associated with oxidative stress.

Key words luteolin; neuroprotection; heme oxygenase-1; apoptosis; oxidative stress; rat brain cortical cell

Oxidative stress, which represents an imbalance between oxidant and anti-oxidant mechanisms, is caused by excessive generation of reactive oxygen species (ROS) such as O2·-, OH·, NO and H2O2.1) This stress provokes the disruption of the structure and function of vital biomolecules including proteins, DNA and lipids, eventually leading to cell death through the induction of apoptosis or necrosis.2,3) The brain is particularly vulnerable to oxidative stress because of its high utilization of oxygen and lack of adequate anti-oxidant defense mechanisms.4) Substantial evidence indicates that oxidative stress is implicated in neuronal cell death and plays a major role in certain neurodegenerative diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), and cerebral stroke.2,5–9) Additionally, the oxidation of xanthine (X) by xanthine oxidase (XO) or xanthine dehydrogenase serves as an important source of ROS, which leads to the onset of cerebral ischemia, inflammation, and neurodegeneration.10–12)

Heme oxygenase (HO)-1, also known as heat shock protein 32, is one of the Phase II detoxification enzymes which in various cell types acts as a key molecule in the cellular maintenance of adaptive survival response to stimuli such as oxidative stress.13,14) HO-1 catalyzes the oxidative cleavage of heme in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen to produce biliverdin, Fe2+ and CO. Biliverdin is subsequently converted to bilirubin under the action of cytosolic enzyme biliverdin reductase.

Both biliverdin and its metabolite bilirubin have been shown to exert potent anti-oxidant effects.15,16)

As neurons have minimal regenerative capabilities, preventing their degeneration is critical to combat disease progression; however, few effective therapies are currently available.17) Because of their beneficial effects on many neurodegenerative diseases, various polyphenolic compounds from natural sources are currently receiving considerable interest and are subject to pharmacological evaluation to scrutinize their neuroprotective activities.18,19) Luteolin [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromene], a bioflavonoid which is known to be present in many types of plants and plant products, possesses diverse biological properties including anti-oxidant, anti-inflammatory, and anticancer activities.20) In keeping with these properties, growing evidence indicates the neuroprotective actions of luteolin against a number of insults. For instance, luteolin was shown to exhibit neuroprotection against kainic acid-induced damage in rats and to improve learning and memory.21) Using cultured cortical neuronal cells, it was reported that luteolin prevented amyloidβ (Aβ125–135)-induced neuronal cell death.22) In a similar type of cultured cells treated with oxidative insult, luteolin was also found to markedly attenuate cytotoxicity and improve mitochondrial function as well as increase endogenous glutathione level and catalase activity.23) However, the precise molecular mechanism(s) underlying the neuroprotective effects

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of luteolin against H$_2$O$_2$-induced oxidative stress in primary cultured rat cortical cells has not yet been clearly elucidated. To address this issue, the present study evaluated effects of luteolin on the oxidative neuronal damage and ROS generation induced by H$_2$O$_2$ or xanthine/xanthine oxidase (X/XO) in the cultured cortical cells. Additionally, the anti-apoptotic activity of luteolin as well as the signaling involved in HO-1 induction and its role in the neuroprotective action was investigated.

**MATERIALS AND METHODS**

**Materials** Minimum essential medium (MEM), fetal bovine serum (FBS), horse serum (HS), and antibiotic–antimycotic agent were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Luteolin and tin protoporphyrin IX (SnPP) was obtained from Toecris Bioscience (Bristol, U.K.). Laminin, cytosine arabinoside, X, XO, H$_2$O$_2$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7'-dichloro-fluorescein diacetate (DCFH-DA), anti-$\beta$-actin and SP600125 were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). SB203580 was procured from Calbiochem (San Diego, CA, U.S.A.) and LY294002, U0126, anti-phospho-Bad (Ser112), anti-Bad, anti-caspase 3, anti-phospho-extracellular signal-regulated kinase (ERK) 1/2, anti-ERK1/2, anti-phospho-p38 mitogen-activated protein kinase (MAPK, Thr180/Tyr182), anti-JNK, anti-phospho-Akt (Ser473), anti-Akt and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-HO-1 antibody was from Enzo Life Sciences, Inc. (Farmingdale, NY, U.S.A.). All other chemicals were of analytical grade.

Tied-tempregn Sprague–Dawley (SD) rats were procured from Daehan Biolink (Chungbuk, Korea). The animals were maintained in an animal house under a controlled temperature (22±2°C) and relative humidity (40–60%) with a 12h light–dark cycle. They were fed a standard chow diet and allowed access to water ad libitum. All experimental methods and procedures as well as the care and handling of the animals were pursued according to the international guidelines (Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, U.S.A.; National Academy Press: Washington D.C., 1996). The rationale, design, and protocols of the animal experiments were approved by the Institutional Animal Ethical Committee of Dongguk University before the study.

**Primary Cultures of Rat Cortical Cells** Primary cultures of rat brain cortical cells were performed as described elsewhere.$^{24-26}$ Briefly, cerebral cortical cells containing neuronal and non-neuronal cells collected from the brain of SD rat embryos at 17d of gestation were seeded on either 35mm dishes (6×10^5 cells/dish), 24-well plates (6×10^5 cells/well) or 96-well plates (2×10^5 cells/well) pre-coated with laminin and poly-l-lysine in MEM supplemented with 5% FBS, 5% HS, and 1% antibiotic–antimycotic agent. The cultured cells were maintained in an incubator at 37°C in a humidified atmosphere of 95% air/5% CO$_2$. The proliferation of non-neuronal cells was arrested by treatment with 10µM cytosine arabinoside after 7d of cell seeding. All experiments were performed after 10–11d of culturing.

**Treatment of Cells and Measurement of Cell Viability** Cortical cells cultured in 24-well plates were washed with N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES)-buffered control salt solution (HCSS) and subjected to oxidative stress by exposure to 100µM H$_2$O$_2$ for 5min or X (0.5mM)/XO (10mM) for 10min in HCSS in the absence or presence of various concentrations of luteolin. The cells were then washed with HCSS and maintained for 18–20h in MEM. In another experiment, where the influence of HO-1 inhibitor SnPP on the neuroprotective effect of luteolin against H$_2$O$_2$-induced neuronal damage was investigated, the cells were treated with 20µM of SnPP for 60min after washing with HCSS.$^{27}$ The cells were then co-treated with H$_2$O$_2$ (100µM) and luteolin for 4h. Following the desired treatments, cell viability was assessed by MTT reduction assay as reported previously.$^{26}$ The absorbance was measured at 550nm using a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, U.S.A.).

**Measurement of Intracellular ROS Generation** The generation of intracellular ROS was assessed by DCFH-DA assay as described previously.$^{26}$ Briefly, the cells were washed with HCSS and exposed to 10µM DCFH-DA in MEM for 30min. Following this treatment, the cells were washed with HCSS and exposed to either 100µM H$_2$O$_2$ or X (0.5mM)/XO (10mM) for 2h in MEM in the absence or presence of various concentrations of luteolin. The determination of intracellular ROS levels was evaluated by fluorescence detection on a SpectraMax M2e microplate reader with an excitation wavelength of 490nm and an emission wavelength of 520nm.

The effect of luteolin on intracellular ROS generation was further evaluated using fluorescence microscopy (Nikon, Tokyo, Japan) under the condition described above. The fluorescence was detected using a fluorescein isothiocyanate (FITC) filter set and the acquired images were processed with the help of Meta imaging system software (Molecular Devices).

**Measurement of DNA Fragmentation** DNA fragmentation was determined using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay kit (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions. Briefly, following the treatments, the cells were fixed in 4% paraformaldehyde for 25min, washed with phosphate buffered saline (PBS), and then permeabilized with 0.2% Triton X-100 for 5min. Following this, the cells were equilibrated with an equilibration buffer for 10min and incubated for 60min at 37°C in terminal deoxynucleotidyl transferase reaction mixture containing a biotinylated nucleotide mix. After this reaction, the cells were immersed in 2× saline sodium citrate solution for 15min and incubated with streptavidin-conjugated HRP for 30min at room temperature. Finally, the cells were reacted with dianimobenzidine and the stained TUNEL-positive cells were identified using a TS-100 inverted microscope (Nikon, Tokyo, Japan) at 400× magnification. The TUNEL-positive cells were counted from four randomly selected fields and expressed as percentages of the total number of cells.

**Western Blotting** Cells were washed with PBS after the desired treatments and lysed in the lysis buffer containing protease inhibitor cocktail (Roche Diagnostic GmbH, Mannheim, Germany). The prepared lysates were centrifuged
at 14000 rpm for 30 min at 4°C and the supernatants were separated for further use in Western blotting. The protein concentrations of the samples were determined using a DC™ protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

The samples were electrophoresed on 10 or 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose or immobilon-P polyvinylidene difluoride (PVDF) membrane. The membranes were incubated overnight at 4°C with the specific primary antibodies in 5% bovine serum albumin after blocking with 5% skim milk for 1.5 h. After washing, the membranes were incubated with appropriate HRP-conjugated anti-IgG secondary antibodies for 1.5 h. Following this, the immunoreactive bands were detected with a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA, U.S.A.) using Clarity™ Western ECL Substrate (Bio-Rad). The intensity of the bands of interest was quantitated using Bio-Rad Image Lab 4.1 software (Bio-Rad).

Statistical Analysis All experiments were carried out individually at least three times. Quantitative data are indicated as the mean± standard error of the mean (S.E.M.) IC50 values were determined by non-linear regression using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). The statistical analysis was conducted by one-way ANOVA followed by Tukey’s post hoc test using Sigma Stat 3.5 software (Systat Software, Point Richmond, CA, U.S.A.). A p<0.05 was considered significant.

RESULTS

Effect of Luteolin on the Oxidative Stress-Induced Neuronal Cell Damage Treatment of cells with H2O2 (100 µM) for 5 min or X (0.5 mM)/XO (10 mU/mL) for 10 min caused approximately 87 and 92% cell death, respectively (Figs. 1A, B). To evaluate the protective effects of luteolin against oxidative stress, neuronal cells were exposed to various concentrations of luteolin prior to treatment with H2O2 or X/XO. As can be seen, luteolin treatment at concentrations of 3–30 µM abruptly and dramatically increased the viability of the H2O2- or X/XO-treated cells almost up to that of control cells (Figs. 1A, B). Luteolin, at the concentration of 1 µM, was not effective against H2O2- or X/XO-induced neuronal damage.

Effect of Luteolin on the Oxidative Stress-Induced ROS Generation Treatment of cells with H2O2 (100 µM) or X (0.5 mM)/XO (10 mU/mL) for 2 h increased the intracellular ROS production to approximately 223 and 241%, respectively, compared to that of control cells (Figs. 2A, B). Co-exposure of the H2O2-treated cells to 0.3–30 µM luteolin significantly attenuated the ROS generation in a concentration-dependent manner (Fig. 2A). The calculated IC50 value was 0.65 µM. This anti-oxidative effect of luteolin against H2O2 insult was further confirmed by epifluorescence study, which revealed a marked inhibition of the H2O2-induced ROS generation by luteolin at 3 µM concentration (Fig. 2C). Co-exposure of the X/XO-treated cells to luteolin also concentration-dependently decreased ROS production (Fig. 2B), with the IC50 value of 7.66 µM.

Effect of Luteolin on the Oxidative Stress-Induced Apoptosis We next examined the effects of luteolin on oxidative stress-induced apoptosis in the cultured neuronal cells. As illustrated in Figs. 3A and B, treatment of cells with 100 µM H2O2 for 4 h significantly decreased the phosphorylation of Bad at Ser112 and increased the cleavage of caspase 3, the hallmarks of apoptotic events. In keeping with this, TUNEL assay also revealed that exposure of neuronal cells to 100 µM H2O2 for 2 h led to the appearance of a large number of TUNEL-positive cells (see arrows in Fig. 3C), indicating the fragmentation of DNA, a crucial event in apoptosis. Co-exposure of H2O2-treated cells to luteolin at 3 µM significantly increased the Ser112 phosphorylation of Bad and inhibited the cleavage of pro-caspase 3. The appearance of TUNEL-positive cells was decreased by the exposure to luteolin at both 3 and 10 µM concentrations, confirming the anti-apoptotic action of this flavonoid against oxidative insult (Figs. 3A–D).

Up-Regulation of HO-1 Expression by Luteolin and Its Involvement in Neuroprotective and Anti-apoptotic Effects To further understand the molecular mechanism(s) underlying-
Involvement of MAPK and Akt Pathways in Luteolin-Induced HO-1 Expression

To elucidate the possible signaling pathway(s) leading to the luteolin-mediated up-regulation of HO-1 expression, the effect of this compound on MAPKs and Akt signaling was investigated. It was found that luteolin significantly increased the phosphorylation of ERK, p38 MAPK, JNK and Akt during the treatment period of 10–120 min (Figs. 5A, B). Phosphorylation of ERK, JNK and Akt was observed at earlier time points (10–30 min), whereas phosphorylation of p38 MAPK was obvious at later time points (60–120 min).

To examine whether the phosphorylation of these kinases is essential for the luteolin-induced HO-1 expression, selective protein kinase inhibitors were employed; namely, U0126 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), and LY294002 (phosphatidylinositol 3-kinase (PI3K) inhibitor). As shown in Figs. 5C and D, luteolin-induced HO-1 expression was significantly inhibited by SB203580, SP600125 and LY294002, but not by U0126. In agreement with these findings, HO-1 induction in the H_{2}O_{2}+luteolin-treated cells was also significantly inhibited.

Fig. 2. Inhibition of Oxidative Stress-Induced ROS Generation by Luteolin

Cells were treated with 10 \mu M DCFH-DA for 30 min and then exposed to 100 \mu M H_{2}O_{2} (A, C) or 0.5 \mu M X/10 mU/mL XO (B) for 2 h in the absence or presence of luteolin at the indicated concentrations. Intracellular ROS generation was assessed as described in Materials and Methods. Each data point represents the mean±S.E.M. from at least three measurements performed in duplicate (* p<0.05 vs. control; # p<0.05 vs. H_{2}O_{2} or X/XO-treated cells).

Involving the neuroprotective effects of luteolin, its influence on the expression of HO-1, an enzyme with potent anti-oxidant activity, was investigated. Western blotting results demonstrated an up-regulation of HO-1 expression in response to treatment with 3 \mu M luteolin (Figs. 4A, B). The effect was found to be significant and time-dependent at 3–6 h of treatment with luteolin. To further confirm whether the neuroprotective and anti-apoptotic effects of luteolin are mediated through the up-regulated HO-1, SnPP, a well-known inhibitor of HO-1, was employed. It was found that the pre-exposure of cultured cells to SnPP significantly attenuated the protective effects of luteolin against H_{2}O_{2}-induced neuronal damage. The inhibition by SnPP was more prominent in the cells treated with luteolin at the concentration of 3 \mu M than 10 \mu M in the presence of H_{2}O_{2} (Fig. 4C). Similarly, the anti-apoptotic effect of luteolin was markedly blocked by SnPP, reducing the number of TUNEL-positive cells (Figs. 4D, E). These results strongly corroborate the hypothesis that luteolin combats oxidative stress in neuronal cells, at least in part, via the up-regulation of HO-1 expression.
by SB203580, SP600125 and LY294002, but not by U0126. Collectively, these results suggest that the induction of HO-1 expression by luteolin involves p38 MAPK, JNK and Akt signaling pathways.

**DISCUSSION**

Oxidative stress, which is triggered by an imbalance in the generation of ROS and the inability of biological systems to detoxify these species and repair the resulting damage, plays a vital role in the onset and development of a number...
of diseases. The involvement of oxidative stress in neuronal cell death, ultimately leading to certain neurodegenerative diseases such as AD, PD, and cerebral stroke, is well documented.\(^2,5–9,29\)

Neuroprotection and neurorescue can be considered promising therapeutic approaches to combat neurodegenerative diseases, as they not only delay the progression of the disease but also improve the disease conditions. A large number of plants and plant products have long been used as traditional neuroprotective medicines to treat cognitive disorders, including AD and other memory-related disorders.\(^30\) For the past few years, much attention has been focused on polyphenolic compounds, the secondary metabolites of plants, because of their anti-oxidant and free radical scavenging activities as well as beneficial effects on learning and memory, cognitive impairment and many neurodegenerative diseases.\(^{18,19}\)

Luteolin, a member of the bioflavonoid family, is known to be present in several types of plants and plant products, including fruits, vegetables, and medicinal herbs. Previous studies have revealed the prominent anti-oxidative effects and radical scavenging activities of luteolin against damage induced by oxidative stress in various cell types including H9c2 cells, SH-SY5Y cells, primary neurons and RAW264.7 cells.\(^{23,31–33}\)

In keeping with these, a number of studies have demonstrated that luteolin possesses a wide variety of beneficial pharmacological properties including anti-inflammatory and anti-cancer activities.\(^{34,35}\) These pharmacological activities of luteolin are connected with its anti-oxidative effects and free radical scavenging activities where the active polyphenolic hydroxyl group plays an important role. Emerging
the neuroprotective activities of this flavonoid. The present study, using primary cultured rat cortical cells, demonstrated a marked neuroprotective effect of luteolin against \textit{H}_2\textit{O}_2- or \textit{X/O}-induced oxidative insults. Based on the results presented in this study, this flavonoid at concentrations as low as 3 \( \mu \text{M} \) abruptly increased the viability of \textit{H}_2\textit{O}_2- or \textit{X/O}-treated cells to the same level as the control cells (Figs. 1A, B). In parallel, luteolin was found to markedly inhibit ROS production in both \textit{H}_2\textit{O}_2- and \textit{X/O}-treated cells, demonstrating its potent anti-oxidant activity (Figs. 2A–C). Their calculated IC\textsubscript{50} values indicate that the \textit{H}_2\textit{O}_2-induced ROS generation was more potently inhibited than the \textit{X/O}-induced ROS (Figs. 2A, B), implying preferable inhibition of \textit{H}_2\textit{O}_2 or hydroxyl radicals by luteolin compared to superoxide radicals. These findings are in agreement with a previous study revealing luteolin as a potent neuroprotectant against

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**Fig. 5. Luteolin-Induced Phosphorylation of ERK, p38 MAPK, JNK or Akt and Its Role in HO-1 Expression**

Cells were exposed to 3 \( \mu \text{M} \) luteolin for the indicated time periods and the phosphorylation of ERK, p38 MAPK, JNK or Akt was evaluated by Western blotting as described in Materials and Methods (A). Cells were exposed to selective protein kinase inhibitors, including U0126 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), and LY294002 (PI3K inhibitor) for 60 min and then co-treated with 100 \( \mu \text{M} \) \textit{H}_2\textit{O}_2 + 3 \( \mu \text{M} \) luteolin in the presence of the respective inhibitor for 4 h (C). Levels of HO-1 expression were evaluated by Western blotting as described in Materials and Methods. The intensities of the respective bands quantified by densitometric analyses were normalized to total ERK, p38 MAPK and JNK/\( \beta \)-actin or \( \beta \)-actin (B, D). Each data point represents the mean \pm S.E.M. from at least three measurements performed in duplicate (B, *\( p < 0.05 \) vs. control, D, *\( p < 0.05 \) vs. control; *\( p < 0.05 \) vs. \textit{H}_2\textit{O}_2-treated cells; $\text{p} < 0.05 \) vs. cells treated without inhibitor).
ROS-induced neurotoxicity in neuronal cells.23 Apoptosis, a form of programmed cell death where a stimulus triggers a cascade of events that orchestrates the destruction of cells, plays an important role in the development and progress of neurodegenerative diseases.17,36–38 Consequently, the inhibition of apoptosis offers a useful strategy in the prevention and treatment of diverse neurodegenerative diseases.36,38 The roles of Bad and caspase 3 as key players in the apoptotic signaling pathway are well documented.39 Bad, a pro-apoptotic member of the Bcl-2 protein family forms a complex with Bcl-xL, an anti-apoptotic member of the Bcl-2 protein family. Bcl-xL is unable to exert its anti-apoptotic effects in its bound form. When Bad is phosphorylated at Ser112, Ser136 or Ser155, it is trapped by the 14-3-3 protein in the cytosol and thereby prevented from binding to Bcl-2/Bcl-xL, which ultimately promotes cell survival.36,40,41 The activation of caspase 3 initiates the execution pathway for apoptosis, leading to DNA fragmentation.40 In this study, the exposure of cultured cells to H2O2 caused a marked decrease in the Ser112 phosphorylation of Bad and an increase in the active caspase 3, which ultimately led to an increased number of TUNEL-positive cells, vital hallmarks of apoptosis. This is in keeping with the action of H2O2 as an apoptotic inducer.42,43 The present study revealed that luteolin increased the Ser112 phosphorylation of Bad and decreased the activation of caspase 3 as well as markedly reduced DNA fragmentation in the H2O2-treated cells, suggesting that the prevention of apoptotic events may largely contribute to the neuroprotective effects of this compound against oxidative insult (Figs. 3A–D).

HO-1, one of the Phase II detoxification enzymes, plays a critical role in the cellular maintenance of adaptive survival response to oxidative stress.13,14 It has been reported that several plant-derived polyphenolic compounds can exert neuroprotective action through the regulation of HO-1 expression.44 Keeping this in consideration, the influence of this flavonoid on HO-1 expression was investigated in the H2O2-treated cells in order to further understand the molecular mechanism(s) underlying its neuroprotective activity. Luteolin caused a marked, time-dependent induction of HO-1 expression (Figs. 4A, B). In order to investigate whether the up-regulation of HO-1 expression by luteolin is associated with its neuroprotective action, we examined the cell viability and appearance of TUNEL-positive cells in the absence or presence of SnPP, a well-known selective HO-1 inhibitor. Notably, the increase in the cell viability of the H2O2-treated cells by luteolin was significantly attenuated by the treatment with SnPP (Fig. 4C). Similarly, inhibition of DNA fragmentation by luteolin was markedly blocked by SnPP (Figs. 4D, E), strongly supporting that up-regulated HO-1 plays a critical role in the neuroprotective effect of luteolin in the cultured cortical neurons.

The roles of Akt and MAPK pathways in cell survival and apoptosis are well documented.45,46 Activation of MAPKs or Akt is known to be involved in the upstream signaling pathway for the expression of HO-1 in neuronal and other cell types.47,48 It has been reported that berberine, an alkaloid derived from Coptis chinensis, induces the stimulation of Akt and p38 MAPK, which is linked to HO-1 expression and neuroprotection in SH-SY5Y cells.49 Additionally, geniposide, an iridoid glycoside from the fruit of Gardenia jasminoides, has been found to trigger the expression of HO-1 in hippocampal neurons through PI3K/nuclear factor-E2-related factor 2 (Nrf2)-signaling, which largely mediates the anti-oxidant action of this compound.50 The present study elucidated the probable signaling cascades that lead to luteolin-induced up-regulation of HO-1 expression in untreated or H2O2-treated cultured neurons. Luteolin was revealed to time-dependently increase the phosphorylation of ERK, p38 MAPK, and JNK as well as Akt, suggesting the possible involvement of these protein kinases in luteolin-induced HO-1 expression (Figs. 5A, B). Pretreatment with the respective specific inhibitors of these kinases revealed that p38 MAPK, JNK, and Akt are the upstream signaling pathways involved in the up-regulation of HO-1 expression induced by luteolin. In contrast, phosphorylation of ERK did not appear to mediate the luteolin-induced HO-1 expression, since the MEK inhibitor U0126 was unable to reduce HO-1 induction (Figs. 5C, D). Interestingly, we observed that the basal HO-1 activity was also significantly inhibited by SP600125 itself at the concentration tested in this study (Figs. 5C, D), raising the possibility that the basal JNK activity rather than luteolin-induced activation of this kinase might be important for the HO-1 induction. However, the extent of inhibition of the luteolin-induced HO-1 up-regulation by SP600125 is more dramatic than the inhibition of the basal HO-1 expression (Fig. 5D). Therefore, we concluded that JNK pathway, along with other pathways such as p38 and Akt, is involved in the HO-1 up-regulation by luteolin. Our findings are in agreement with the recent report,51 showing in RAW264.7 cells that luteolin activated HO-1 expression.
Akt is involved in the luteolin-induced up-regulation of HO-1. We demonstrated that the phosphorylation of p38 MAPK, JNK, and Akt is involved in HO-1 expression in primary cultured rat cortical cells. We also demonstrated that the phosphorylation of p38 MAPK, JNK, and Akt is involved in HO-1 expression (Fig. 6). These findings imply that luteolin may serve as a potential pharmacological intervention for neurendinegenerative diseases associated with oxidative stress.

Acknowledgments We thank Dr. S. Bose for assistance in the manuscript preparation. This research was funded by the GRRC program of Gyeonggi province (GRRC-DONGGUUK 2016-B01 and B03).

Conflict of Interest The authors declare no conflict of interest.

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