Luteolin-induced apoptosis through activation of endoplasmic reticulum stress sensors in pheochromocytoma cells

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Abstract. Luteolin [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone] is an active flavonoid compound from *Lonicera japonica* (*Caprifoliaceae*). Luteolin inhibits tumor cell proliferation, inflammatory and oxidative stress better, when compared with other flavonoids. In the present study, it was demonstrated that luteolin induces typical apoptosis in PC12 cells (derived from a pheochromocytoma of the rat adrenal medulla) accompanied by DNA fragmentation and formation of apoptotic bodies. In addition, luteolin regulates expression of the endoplasmic reticulum (ER) chaperone binding immunoglobulin protein, activating ER stress sensors (eukaryotic initiation factor 2α phosphorylation and X-box binding protein 1 mRNA splicing) and induced autophagy. The results indicated that luteolin induces the upregulation of the unfolded protein response pathway through the ER stress sensors, which helps as an influential regulator for the apoptosis pathway in PC12 cells. The results suggested that the understanding of the molecular mechanisms underlying luteolin-induced apoptosis may be useful in cancer therapeutics, chemoprevention and neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease.

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Introduction

Cells maintain their numbers by dividing, repairing, growing and dying through the cell cycle under normal conditions. However, an abnormal recovery mechanism following damage to cells may result in cell death. These cells may be genetically modified, grow excessively and become a malfunctioning cell mass, or cancer (1,2). Cancers are one of the most common conditions globally, with >1.5 million treated for cancer in 2010, according to the US National Cancer Institute (Rockville, MD, USA). The South Korean National Cancer Information Center reported that ~1 in every 3 people will develop cancer at some point in their lifetime (3). The incidence of all patients with cancers was estimated to be 445.3/100,000 people in 2012.

Cancer cells divide rapidly, proliferate and invade surrounding tissues and organs, which eventually malfunction and are destroyed. Because of these cancer cell characteristics, it is difficult to treat cancer by only killing cancer cells without side effects affecting normal cells. Cancer treatment is divided into chemotherapy, radiation therapy and surgery (4). Chemotherapy involves drugs that kill or weakens cancer cells directly, however it is expensive, and the courses are long in duration. Chemotherapy often causes a variety of side effects, such as excruciating pain, anemia, decreased numbers of white blood cells and platelets, vomiting, diarrhea, reproductive disorders and chronic fatigue (5,6). Thus, new methods to improve the clinical response to cancer chemotherapy with drugs that have fewer or no side effects are required.

Flavonoids are secondary plant metabolites and are biologically active polyphenolic compounds (7,8). Among the various types of flavonoids, luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone) is a flavone in many substances, including celery, broccoli, green pepper, parsley and thyme (9). Several cellular and molecular biology studies have demonstrated that luteolin may possess anticancer activities (10,11).

Luteolin has a strong inhibitory effect on the nuclear factor-κB pathway, which is continuously active in cancer cells (12). Proliferation of many kinds of cancer cells has been previously inibited by luteolin, including lung (A549), colon (HCT116), liver (HepG2), breast (MCF7/6), tongue (SCC-4), cervix (HeLa) and leukemia (HL-60) cells (13-19). Nevertheless, inhibiting cancer cell proliferation is a block in the development of new anticancer agents, because the details of the molecular mechanism remain unclear. Therefore, the authors tested the rat PC12 adrenal medulla pheochromocytoma cell line, which characteristic of neuroblastic and eosinophilic cells, as a model to examine the induction of endoplasmic reticulum (ER) stress-mediated apoptosis.

The ER is a membrane-bound intercellular organelle where lipid biosynthesis, post-translational modification, folding, processing and trafficking of secreted and membrane-bound proteins occurs (20). ER molecular chaperones serve central roles, and the binding immunoglobulin protein (BiP) is the most representative chaperone; therefore, it can be used as an ER stress marker. The cellular response to ER stress is called the unfolded protein response (UPR), in which three ER stress sensors, inositol requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6), are downstream components of ER chaperones (21-24). Although luteolin-induced ER stress has been reported, little is known about the mechanisms of luteolin-induced apoptosis. In the current study, PC12 cells were used to understand the molecular mechanism of luteolin-induced activation of the UPR pathway. The results hope to provide clues for the therapeutic effects of luteolin on apoptosis through ER stress.

Materials and methods

Cell culture. PC12 cells were purchased from American Type Culutre Collection (cat. no. CRL-1721; Manassas, VA, USA) and cultured on collagen-coated flasks in 85% RPMI-1640 medium, supplemented with 25 mM HEPES buffer, 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 g/l d-(+)-glucose, 25 μg/ml streptomycin and 25 U/ml penicillin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were maintained in a humidified incubator at 37˚C in a 5% CO_2 atmosphere, and the medium was changed every 2 days. The cells were rinsed with 1X DPBS and detached with 0.25% trypsin/EDTA (both Gibco; Thermo Fisher Scientific, Inc.). After centrifugation at 1,000 x g for 5 min, the cells were subcultured in 25 cm^2 flasks using 1:2-1:4 subcultivation ratios and were photographed every 24 h with an inverted microscope. Cells were passaged twice weekly. The 80% confluent monolayer of PC12 cells was treated with luteolin at the indicated doses and times. Total RNA from cultured cells was extracted using an RNA isolation reagent (TRI-Reagent; Ambion; Thermo Fisher Scientific, Inc.) and measured using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The resulting RNA was used for the following reverse transcription-quantitative polymerase chain reaction experiments.

MTT assay. Cell viability measurements by MTT assay. Growth and viability of PC12 cells were determined using MTT from Sigma-Aldrich; Merck KGaA (Darmadst, Germany). The cells were seeded in 96-well plates (at 60 to 80% confluency) and treated with luteolin for 0 (control), 4 and 16 h. MTT solution (0.5 mg/ml) was added to each well, and the plates were incubated for an additional 4 h at 37˚C. Following removal of the medium, the formazan crystals were solubilized in DMSO (Sigma-Aldrich; Merck KGaA). Color development was monitored at 595 nm with a reference wavelength of 650 nm using the Sunrise™ microplate reader (Tecan Trading AG, Männedorf, Switzerland).

Hoechst 33342 staining. Following treatment with luteolin, PC12 cells were incubated for 30 min with Hoechst 33342 (Molecular Probes; Thermo Fisher Scientific, Inc.) loading dye and washed three times in ice-cold 1X PBS. Following staining for 10 min, the stained cells were monitored using a fluorescence microscope (Axio Scope A1; Zeiss GmbH, Jena, Germany) at 340 nm.

DNA fragmentation assay. Cells were lysed in 100 μl 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. Following centrifugation for 5 min at 16,000 x g, the supernatant was treated with RNase A and proteinase K (Promega Corporation, Madison, WI, USA). Subsequently, 20 μl of 5 M NaCl and 120 μl isopropanol were added and kept on ice for 1 h. Following centrifugation for 15 min at 16,000 x g, the DNA pellets were dissolved in 20 μl TE buffer (10 mM Tris-HCl and 1 mM EDTA). The DNA samples were loaded onto a 0.7% agarose gel and observed using a UV source after ethidium bromide (Sigma-Aldrich; Merck KGaA) staining.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR conditions included 30 cycles of the following: 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 1 min (10 min in the final cycle) using the below primers with *Taq* DNA polymerase (Solgent Co., Ltd., Daejeon, Korea). The RT-PCR primers were supplied by Bioneer Corporation (Daejeon, Korea). All chemicals were purchased from Sigma-Aldrich; Merck KGaA. The RT-PCR primers are as follows: IRE1 forward, 5'-ACCACCAGTCCATCG CCATT-3' and reverse, 5'-CCACCCTGGACGGAAGTT TG-3'; ATF6 forward, 5'-CTAGGCCTGGAGGCCAGG TT-3' and reverse, 5'-ACCCTGGAGTATGCGGGTTT-3'; PERK forward, 5'-GGTCTGGTTCCTTGGTTTCA-3' and reverse, 5'-TTCGCTGGCTGTGTAACTTG-3'; BiP forward, 5'-AGTGGTGGCCACTAATGGAG-3' and reverse, 5'-TCT TTTGTCAGGGGTCGTTC-3'; β-actin forward, 5'-ACA TCAAATGGGGTGATGCT-3' and reverse, 5'-AGGAGA CAACCTGGTCCTCA-3'. The figure presented the results of a representative experiment from three experiments with different samples.

Western blot analysis. PC12 cells were scraped and lysed by adding SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol and 125 mM DTT. Protein concentration was determined as described previously (25). Protein (15 μg) was separated by 10% SDS-PAGE gel electrophoresis. The proteins were transferred to a nitrocellulose membrane, and the membranes were blocked by the 5% skim milk in 0.1% TBST (TBS with 0.1% Tween-20) for 1 h at room temperature

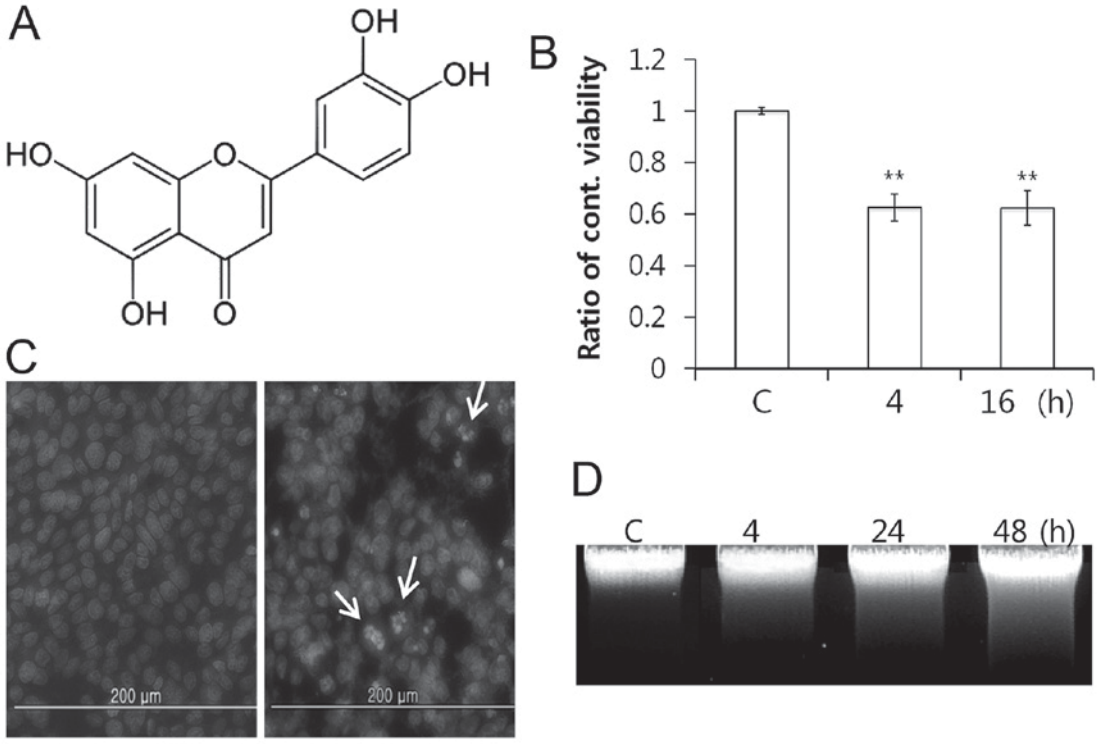

Figure 1. Luteolin induces apoptosis in PC12 cells. (A) The molecular structure of luteolin (chemical formula, $C_{15}H_{10}O_6$; molar mass, 286.24 g·mol^{-1}). (B) PC12 cells were treated with 100 μM luteolin for 4 or 16 h. Cell viability was measured by MTT assay. Data is presented as the mean ± standard deviation. **P<0.01 vs. C (control). (C) The cells were treated with 100 μM luteolin for 24 h and stained with Hoechst 33342 solution to detect formation of apoptotic bodies. Stained nuclei were observed under a fluorescent microscope using a blue filter. (D) To confirm DNA fragmentation, cells were treated with 100 μM luteolin for 4, 24, and 48 h. DNA was resolved on a 1.5% agarose gel and visualized with ethidium bromide.

and incubated with the primary antibodies (all 1:1,000 dilution) overnight at 4˚C. The rabbit anti-eIF2α antibody (cat. no. sc-133132), eIF2α-P antibody (cat. no. sc-133132p) and goat anti-actin antibody (cat. no. sc-1616-r) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The horseradish peroxidase-conjugated anti-rabbit (cat. no. sc-2004), anti-goat (cat. no. sc-2020) and anti-mouse (cat. no. sc-2005) IgG secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. and were reacted with a 1:1,000 dilution for 1 h at room temperature. Goat anti-actin antibody was used to standardize the quantity of sample proteins. The mouse anti-ATF6 antibody (cat. no. NBP1-40256) was obtained from Novus Biologicals, LLC (Littleton, CO, USA). The blots were developed using an enhanced chemiluminescence western blotting detection system kit (Amersham; GE Healthcare Life Sciences, Chalfont, UK). Experiments were performed in triplicate and the protein bands were quantified using ImageJ software (version 1.48; https://imagej.nih.gov/ij/).

Statistical analysis. All statistical analyses were performed using the SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Analysis of variance and Tukey-honest significant difference post hoc tests were performed to analyze the statistical significance. P<0.05 was considered to indicate a statistically significant difference. Data are expressed as the mean ± standard deviation, or as median values, in accordance with Gaussian distribution. All variables included in the regression analysis respected a linear distribution; when necessary, variables were linearized and checked for normality.

Results and discussion

Luteolin (Fig. 1A) is a flavone present in parsley, artichoke, celery and green pepper (26,27). These plants have long been used in traditional medicine to treat a broad range of diseases. Luteolin inhibits growth of many cell types (28). The preliminary results using PC12 cells demonstrated morphological differences between luteolin-treated and control cells, as well as an increase in the number of floating cells in the medium (data not shown). This finding suggested that luteolin leads to cell death. The authors then tested the effects of 100 μM luteolin on cell viability in the MTT assay following 4 and 16 h of treatment. Luteolin treatment inhibited the PC12 cell growth time-dependently, and luteolin-treated cell growth was reduced by half, following 4 h (Fig. 1B). This result suggested that apoptosis was induced based on cell shrinkage and extensive detachment of the cells (29). A total of two experiments were performed to determine whether luteolin induces chromatin condensation and DNA fragmentation, which are hallmarks of apoptosis. As a result, different nuclei were observed in cells treated with and without luteolin following Hoechst 33342 staining (Fig. 1C). Next, inter-nucleosomal DNA fragmentation increased time-dependently, which is the typical ladder pattern of apoptosis (Fig. 1D). These results indicated that luteolin-induced inhibition of cell growth is associated with induction of apoptosis in PC12 cells.

The UPR in mammalian cells comprises three separate ER stress sensors. These are downstream components of ER chaperones, which transmit stress signals from the ER to the

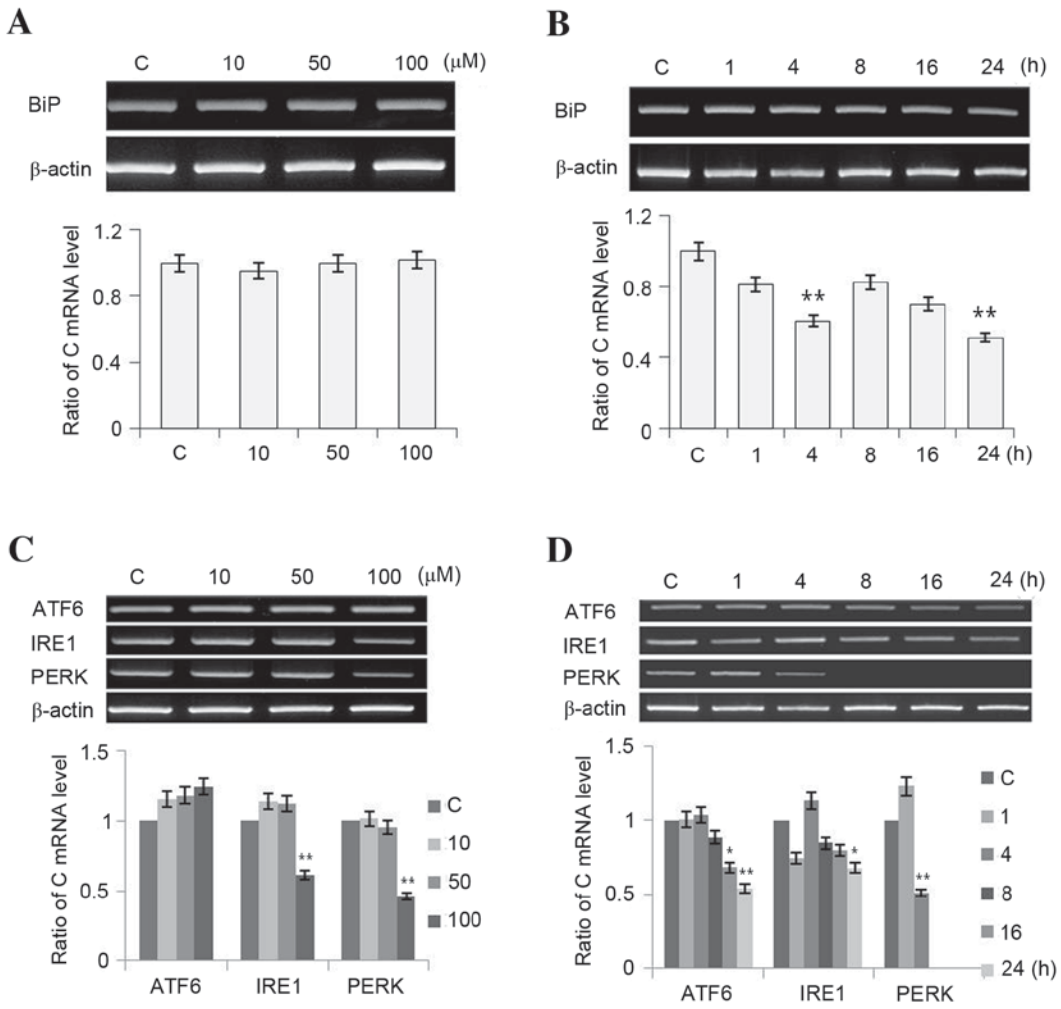

Figure 2. Expression of (A and B) ER chaperones and (C and D) ER stress sensors at different luteolin doses and times. PC12 cells were treated with luteolin (10, 50, 100 and 200 μM; panels A and C) for 1, 4, 8, 16 and 24 h (panels B and D). The revere transcription-polymerase chain reaction results were measured three times. Data is presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. C (control). ER, endoplasmic reticulum; BiP, immunoglobulin heavy-chain binding protein; ATF6, activating transcription factor 6; IRE1, inositol-requiring kinase 1; PERK, PKR (protein kinase regulated by RNA)-like ER-associated kinase; C, control.

nucleus. IRE1 activates the endonuclease domains, which cleave X-box DNA-binding protein (XBP) mRNA, generating an activated form of the XBP1 protein (30). Activating PERK results in phosphorylation of the eIF2α subunit and inhibits translation initiation (31). ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, and the resulting N-terminal cytoplasmic domain subsequently binds to both the ER stress-response element and ATF6 sites, to enhance expression of ER molecular chaperone genes (32,33). Binding immunoglobulin protein (BiP) binds transiently to newly synthesized proteins in the ER when a cell is ER stress-free. However, stimulating ER stress induces interactions with misfolded, underglycosylated and unassembled proteins through activation of ATF6, IRE1 and PERK (34). BiP eventually regulates the equilibrium between cell survival and apoptosis (35). Moreover, BiP is a principal regulator of ER stress signaling and survival in ER-stressed cells. ER stress-induced apoptosis is a key pathological event in various cancer cells (36,37).

To understand whether luteolin-induced apoptosis is associated with ER stress, changes in the expression of BiP ATF6, IRE1 and PERK were evaluated under luteolin-treated conditions in PC12 cells. As presented in Fig. 2, treating PC12 cells with luteolin (10, 50 and 100 μM) for 16 h altered BiP mRNA expression (Fig. 2A), and it decreased in response to 100 μM luteolin following 1, 4, 8, 16 and 24 h of treatment (Fig. 2B). Expression of ATF6 and IRE1 tended to increase, whereas PERK expression decreased as luteolin concentration was increased (Fig. 2C). The expression of all of the ER stress sensors decreased over time reduced (Fig. 2D). In summary, luteolin downregulated transcription of BiP and the ER stress sensors gradually in a time-dependent manner, and upregulated ATF6 and IRE1 mRNA expression in a dose-dependent manner.

Furthermore, the authors investigated activation of the ER stress sensors in response to luteolin treatment, such as ATF6 fragmentation, eIF2-α phosphorylation and XBP1 mRNA splicing. Under ER stress conditions, ATF6 is transported from the ER to the Golgi complex, where it is sequentially cleaved by site 1 and site 2 proteases (38). An anti-ATF6-specific antibody that recognizes a 50 kDa cleaved fragment form of ATF6 was used to understand activation of ATF6 under ER stress conditions. Upon ER stress, PERK phosphorylates eIF2-α

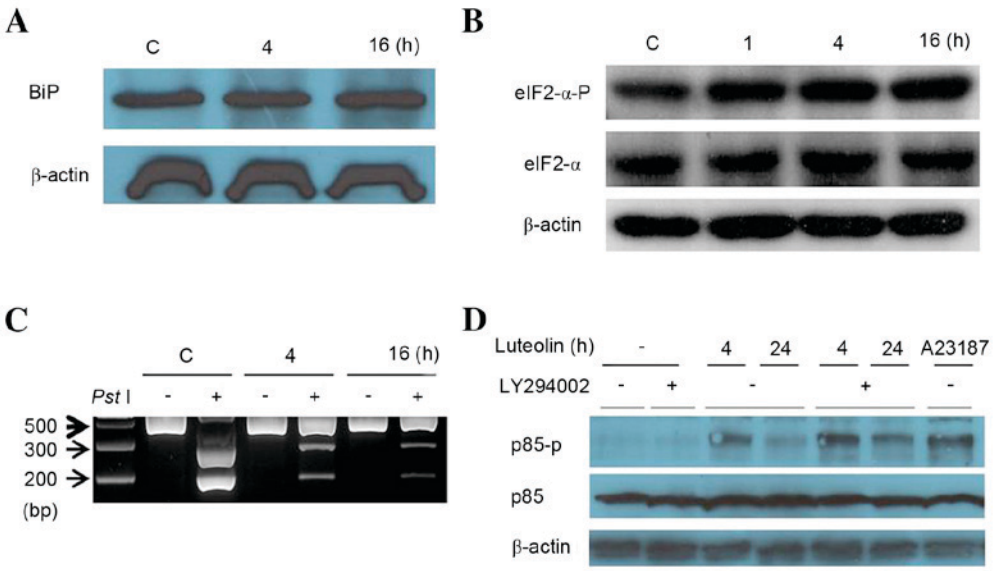

Figure 3. Luteolin controls endoplasmic reticulum stress sensors. (A) Cells were treated with 100 μM luteolin for 4 and 16 h. Cell lysates were subjected to western blotting with mouse anti-ATF6 monoclonal antibody. (B) Western blotting was performed using anti-eIF2-α antibody and eIF2-α-P antibody against cells treated for different times (1, 4 and 16 h). (C) The revere transcription-polymerase chain reaction analysis was performed using mRNAs from Fig. 3A. The resulting PCR product was further digested by *Pst*I to reveal a restriction site that was lost following XBP1 splicing under ER stress. The resulting XBP1 cDNA products were revealed on a 2% agarose gel. Unspliced XBP1 mRNA produced the two lower bands indicated by arrows (upper, 290 bp and lower, 183 bp). The spliced XBP1 mRNA indicated by a bold arrow. (D) Western blotting was performed using a p85 antibody and p85-P antibody against cells treated for 4 and 24 h. ATF6, activating transcription factor; eIF2-alpha, translation initiation factor eIF2-α; eIF2-alpha-P, phosphorylated form of translation initiation factor eIF2α; XBP1, X-box binding protein 1; p85, phosphatidylinositol 3-kinase 85 kDa regulatory subunit alpha; p85-P, phosphorylated form of the phosphatidylinositol 3-kinase 85 kDa regulatory subunit alpha; C, control.

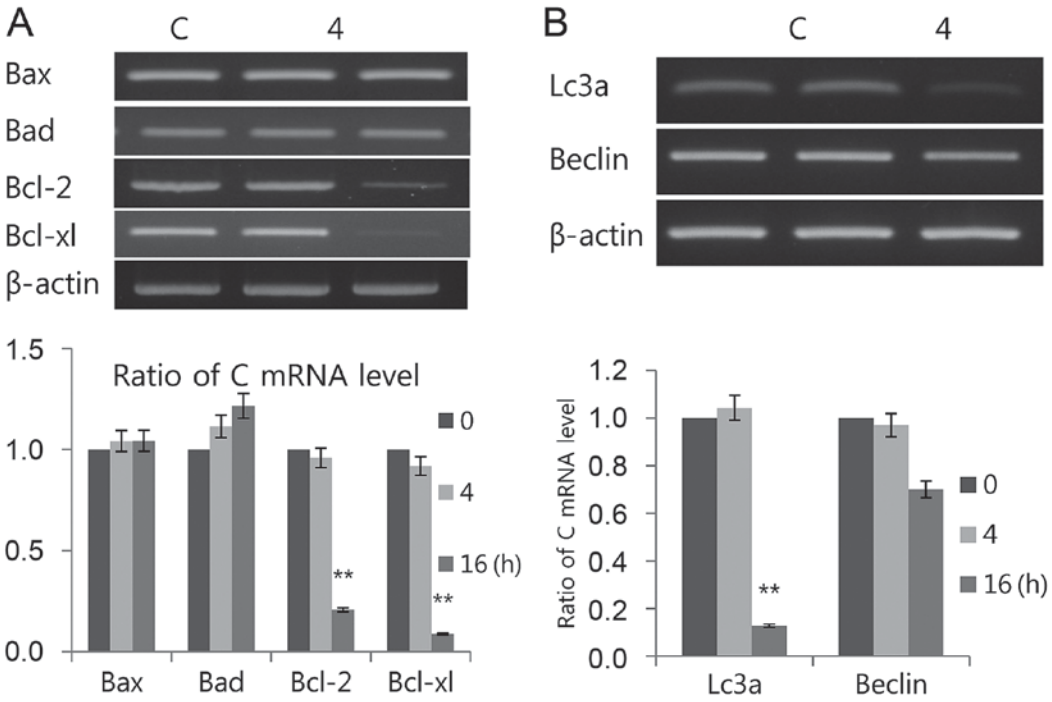

Figure 4. Luteolin induces (A) apoptosis and (B) autophagy. The mRNAs used for both tests were the same as those in Fig. 3A. Bax, Bad, mRNA levels of Bcl-2, Bcl-xl, LC3 and Beclin-1 were measured by RT-PCR. The RT-PCR results were measured three times. Data is presented as the mean ± standard deviation. β-actin was used as the loading control. **P<0.01 vs. C (control). Bax, Bcl-2-associated X; Bad, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2; Bcl-xl, B-cell lymphoma/leukemia-x long; LC3, microtubule-associated protein light chain 3; Beclin-1, coiled-coil moesin-like BCL2 interacting protein; RT-PCR, semiquantitative revere transcription-polymerase chain reaction; C, control.

to reduce biosynthesis of total mRNAs. Thus, we measured eIF2α phosphorylation levels by western blotting to detect ER stress conditions. The ER stress-mediated splicing of XBP1 is a prerequisite for IRE1 activation, which controls the intensity of XBP1 splicing (removing a 26-bp segment from the full-length XBP-1 mRNA) (39). RT-PCR analysis was used to

determine the unspliced and spliced isoforms of XBP1 mRNA. PC12 cells were treated with 100 μM luteolin for 4 and 16 h (Fig. 3). Although luteolin did not induce ATF6 fragmentation (Fig. 3A), it gradually upregulated eIF2-α phosphorylation (Fig. 3B) and XBP1 mRNA splicing (Fig. 3C), suggesting that luteolin triggered the UPR signaling pathway by activating the ER stress sensors, except for ATF6. Phosphotidyl inositol 3-kinase (PI3 K) is a key regulator of many cellular processes, including cell survival, proliferation and differentiation (40). Under resting conditions, PI3K is composed of p85 and p110 (41). p85 interacts with other proteins (small GTPase cdc42, nuclear receptor co-repressor and CD148) that complex and serve significant roles in other cell signal pathways (42). Park *et al* (43) demonstrated that p85 interacts with the spliced form of XBP-1 (XBP-1s), which increases both XBP-1 s activity and the nuclear import of XBP-1s under ER stress conditions. Thus, the authors examined whether phosphorylation of p85 under ER stress conditions is related to luteolin stimulation. As demonstrated in Fig. 3D, short-term treatment (4 h) with luteolin induced higher levels of phosphorylated p85 than those of long-term treatment (24 h). The reason is unclear, but treatment with LY294002 upregulated phosphorylated p85. These data suggested that inhibition of PI3 K is associated with p85 phosphorylation through ER stress.

If early cellular responses fail to maintain ER homeostasis, ER stress activates the UPR to stimulate the apoptosis pathway for cell survival. The authors investigated whether luteolin induces both apoptosis and autophagy through the UPR, as the exact mechanisms of the induction of ER apoptosis and autophagy remain elusive. However, signaling through ER stress trigger several regulators associated with the apoptosis pathway during prolonged ER stress (44). A link between the UPR and autophagy has been presented; as phosphorylation of eIF2α modulate autophagy and ER stress-inducible drugs induce autophagy (45). The present results indicated that luteolin induced transcriptional expression of pro-apoptotic Bax and Bad but decreased anti-apoptotic Bcl-2 and Bcl-xl (Fig. 4A). Luteolin strongly downregulated Lc3 and Beclin transcription levels (Fig. 4B), suggesting that luteolin upregulates apoptosis and downregulates autophagy in PC12 cells. In conclusion, although the underlying mechanism of luteolin in cell death is still unclear, the present study demonstrated luteolin regulates apoptosis and autophagy. Therefore, it may serve as a novel strategy to treat cancer by regulating the UPR signal.

In summary, luteolin treatment markedly induced apoptosis via the unfolded protein response, including ER chaperones and ER stress sensors, in PC12 cells. It is thought that further understanding of the biological mechanisms underlying luteolin-induced apoptosis may be useful in the prevention and treatment of chronic or acute neurological disorders such as Alzheimer's disease and Parkinson's disease as well as their symptoms (back pain) and signs (aphasia), and also neurological syndromes such as Aicardi syndrome.

Acknowledgements

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