Luteolin-induced protection of H_2O_2-induced apoptosis in PC12 cells and the associated pathway

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Abstract. Increasing evidence has indicated that the generation of reactive oxygen species (ROS) contributes to H_2O_2-induced nerve injury. This may result in oxidative stress that leads to cell damage or death. Dietary or pharmaceutical augmentation of the endogenous antioxidant defense capacity is a potential means by which to prevent ROS-induced damage. The aim of the current study was to investigate the effect of luteolin on H_2O_2-induced cell apoptosis in cultured rat pheochromocytoma cells (PC12 cells) and to investigate the role of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway on H_2O_2-induced apoptosis. The results demonstrated that luteolin was able to inhibit the reduction in cell viability induced by H_2O_2. In addition, luteolin reduced ROS generation and lactate dehydrogenase release in H_2O_2-treated PC12 cells. The levels of superoxide dismutase and glutathione peroxidase activity were increased following treatment with luteolin, however malondialdehyde levels were observed to be reduced. Additionally, luteolin increased the Bcl-2/Bax ratio and enhanced Akt phosphorylation. However, these alterations were attenuated by pretreatment with an inhibitor of the PI3K/Akt pathway. In conclusion, luteolin inhibited H_2O_2-induced apoptosis via reducing ROS levels and activating the PI3K/Akt pathway.

Introduction

Oxidative stress is a mechanism commonly implicated in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (1-3). There is increasing evidence that the production of reactive oxygen species (ROS) during oxidative stress leads to mitochondrial dysfunction and apoptosis (4-7). A previous study demonstrated that numerous chemical and physiological inducers of oxidative stress result in apoptosis (7). Among them, H_2O_2 has been extensively used to induce oxidative stress *in vitro* (8). The products of H_2O_2, superoxide and hydroxyl radicals, are the major components of ROS.

A crucial balance between ROS generation and antioxidant defence is important in disease prevention. Antioxidants are able to help reduce neuronal degeneration by preventing the generation of free radicals (9-14). However, the synthetic antioxidants are associated with toxicity and are potential carcinogens (15). Therefore, the development of non-toxic and highly active antioxidant compounds is important.

Luteolin (3,4,5,7-tetrahydroxylflavone) is a component of numerous traditional Chinese medicines, and is a flavonoid compound derived from *Lonicera japonica* Thunb. Luteolin has been demonstrated to possess numerous biological effects, including anti-inflammatory, anti-oxidative and anticarcinogenic activity (16-19). Luteolin has been previously used in pharmacological and clinical practice (20,21). The current study investigated whether luteolin has protective effects against H_2O_2-induced apoptosis in rat pheochromocytoma cells (PC12) cells, and the potential signaling pathways involved were explored.

Materials and methods

Materials. PC12 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell culture medium components were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). H_2O_2 was purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002 was supplied by EMD Millipore (Billerica, MA, USA). Luteolin was obtained from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China) and the purity of the chemical was >98.0%.

Cell culture and treatment. PC12 cells ($1x10^5$) were grown (100 μl/well in 96-well plates) in Dulbecco's modified Eagle's

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medium supplemented with 10% fetal calf serum, 1% penicillin and streptomycin at 37˚C and 5% CO_2 and 95% air for 24 h. Cells were used for experiments during the exponential growth phase. PC12 cells were preconditioned with different concentrations of luteolin (10, 25 and 50 μg/ml) for 1 h, whereas the control cells received 0.9% saline (Beyotime Institute of Biotechnology, Nantong, China) instead. Subsequently, PC12 cells were exposed to H_2O_2 (400 μM, final concentration) for 6 h.

Cell viability assay. Cell viability was determined using the MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. Following H_2O_2 (400 μM) treatment alone or with different concentrations of luteolin for 6 h, cells were incubated with 20 μl MTT (Beyotime Institute of Biotechnology) for 4 h. Cells were pretreated with phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (60 μM) for 1 h at 37˚C to investigate the role of protein kinase B (Akt) in the effect of luteolin (50 μg) on PC12 cells. Absorbance was measured at 570 nm (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and used to calculate the relative ratio of cell viability.

Cytotoxicity assay. Cell death was assessed by measuring LDH release into the medium (22). Following H_2O_2 (400 μM) treatment alone or with different concentrations of luteolin for 6 h, the medium was collected. LDH release was measured according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Measurement of intracellular ROS generation. Intracellular ROS levels were determined using fluorescent 2',7'-dichlorofluorescein (DCF) derived from cell-permeable dichlorodihydrofluorescein diacetate (DCFH-DA) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (23). Following treatment with H_2O_2 (400 μM) alone or with different concentrations of luteolin for 6 h, PC12 cells were incubated with 200 μl medium containing 2 μl 20 mM DCFH-DA solution for 30 min in the dark at 37˚C and 5% CO_2. Subsequently, cells were washed twice with normal medium (PBS; pH 7.4; Beyotime Institute of Biotechnology) and DCF fluorescence was measured with excitation/emission wavelengths of 485/530 nm (BX50-FLA; Olympus Corporation, Tokyo, Japan).

Measurement of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels. Cells were harvested by centrifugation at 1,380 x g at 4˚C for 5 min, washed with cold phosphate-buffered saline (PBS; Gibco Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) twice and homogenized in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM PMSF. The supernatant was then collected. The levels of SOD, GSH-Px and MDA were measured according to the manufacturer's instructions of the respective kits (Nanjing Jiancheng Bioengineering Institute).

Western blotting. Following H_2O_2 (400 μM) treatment alone or with different concentrations of luteolin for 6 h, PC12 cells were washed with cold PBS and homogenized in lysis buffer containing proteinase inhibitors. Following measurement of protein levels using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology), protein was mixed with 5X SDS sample buffer. Subsequently proteins were separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% fat-free milk for 2 h at room temperature, the membranes were incubated overnight at 4˚C with polyclonal antibodies specific to Akt (anti-mouse; 1:1,000 dilution; cat. no. SAB4500797; Sigma-Aldrich), phosphorylated Akt (p-Akt; anti-mouse, 1:1,000 dilution; cat. no. SAB4301414; Sigma-Aldrich), Bcl-2 (anti-mouse; 1:1,000 dilution; cat. no. SAB1305653; Sigma-Aldrich), Bax (anti-mouse; 1:1,000 dilution; cat. no. B3428; Sigma-Aldrich) and β-actin (anti-mouse; 1:1,000 dilution; cat. no. A1978; Sigma-Aldrich). Subsequently, the membranes were incubated with the corresponding secondary antibodies (anti-rabbit; 1:1,000 dilution; cat. no. SE7; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at room temperature for 2 h. The blots were visualized using enhanced chemiluminescence-plus reagent (EMD Millipore), and analyzed using LabImage software, version 2.7.1 (Kapelan GmbH, Halle, Germany).

Statistical analysis. All the experiments were performed a minimum of three times. Values are presented as the mean ± standard deviation. Differences between groups were analyzed using a one-way analysis of variance with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA), followed by Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of luteolin on cell viability in PC12 cells. In order to determine the working concentration of luteolin, PC12 cells were treated with luteolin, from which three concentrations of luteolin (10, 25 and 50 μg/ml) were selected for subsequent experiments. The MTT assay indicated that the percentage of viable cells following treatment with 400 μM H_2O_2 was 22.2±3.1% (Fig. 1A). Following pretreatment with 10, 25 and 50 μg/ml luteolin, cell viability was 30.29±2.1, 45.6±4.7% and 49.4±5.3, respectively. These results indicate that luteolin is able to attenuate H_2O_2-induced cytotoxicity in PC12 cells.

Effect of luteolin on LDH release in PC12 cells. LDH release was used to measure the level of cell death, and compared with the control group, LDH release from cells treated with 400 μM H_2O_2 was 181.5±4.2%. Following pretreatment with different concentration of luteolin, LDH release was 167.2±3.3, 140.3±2.7% and 112.6±5.1, respectively, compared with the control group. These results indicate that luteolin is able to attenuate H_2O_2-induced cytotoxicity in PC12 cells (Fig. 1B).

Effect of luteolin on ROS generation in PC12 cells. The effect of luteolin on H_2O_2-induced ROS generation in PC12 cells was measured. It was observed that treatment of the cells with 400 μM H_2O_2 increased the generation of ROS (Fig. 2). However, the increased ROS generation was significantly

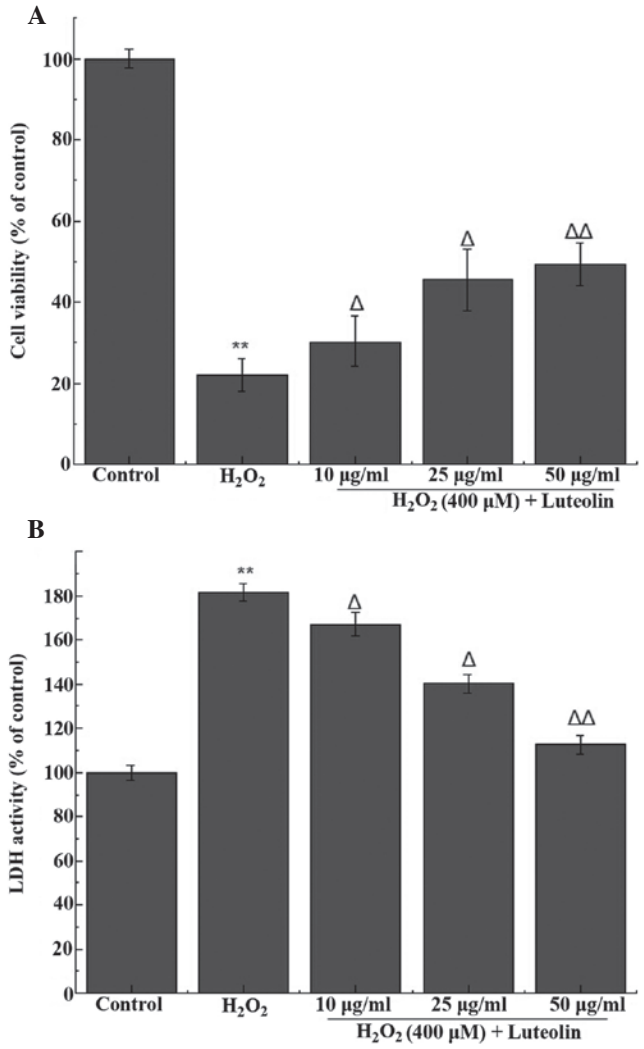

Figure 1. (A) Effect of luteolin on cell viability. (B) Effect of luteolin on LDH activity induced by H_2O_2 in PC12 cells. $^{**}P<0.01$ vs. control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ vs. H_2O_2 alone group. LDH, lactate dehydrogenase; PC12, rat pheochromocytoma cells.

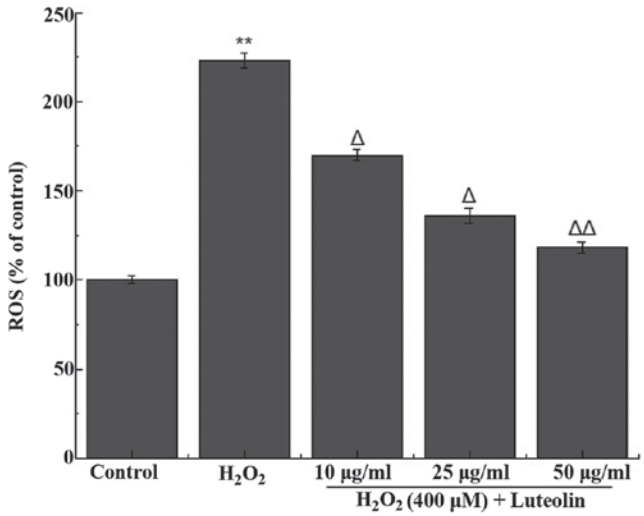

Figure 2. Effect of luteolin on the reduction in ROS generation induced by H_2O_2 in PC12 cells. $^{**}P<0.01$ vs. control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ vs. H_2O_2 alone group. ROS, reactive oxygen species; PC12, rat pheochromocytoma cells.

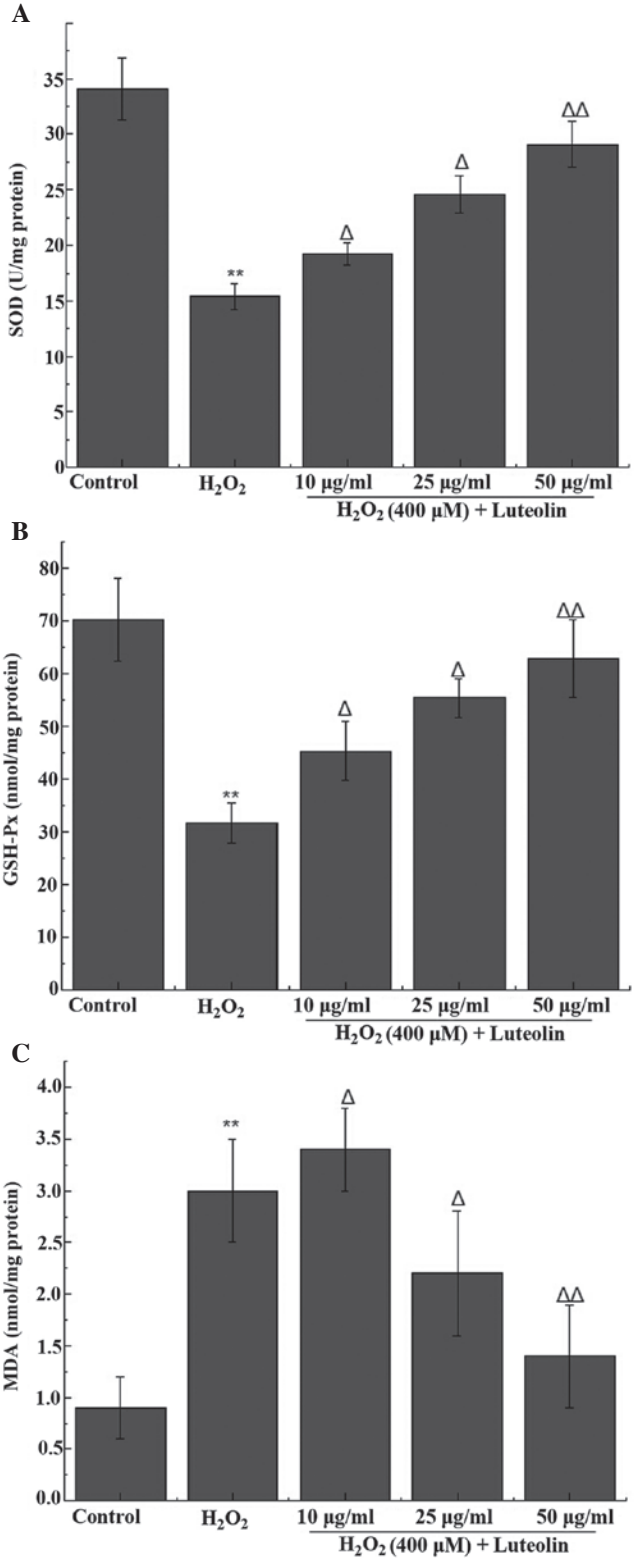

Figure 3. Effect of luteolin on the levels of (A) SOD, (B) GSH-Px and (C) MDA in PC12 cells. $^{**}P<0.01$ vs. control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ vs. H_2O_2 treated alone group. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PC12, rat pheochromocytoma cells.

reduced following pretreatment of the cells with different concentrations of luteolin.

Effect of luteolin on SOD, GSH-Px and MDA levels in PC12 cells. The activity of the antioxidant enzymes (SOD

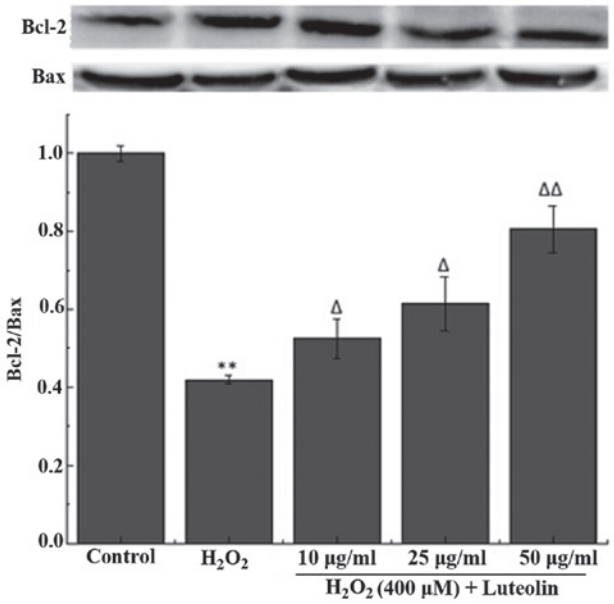

Figure 4. Effect of luteolin on the Bcl-2/Bax ratio induced by H_2O_2 in PC12 cells. The ratio was calculated using LabImage software (version 2.7.1) from the corresponding optical densities. $^{**}P<0.01$ vs. control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ vs. H_2O_2 treated alone group. PC12, rat pheochromocytoma cells.

and GSH-Px) and the end product of oxidation (MDA) were measured in the PC12 cells. The results indicated a significant reduction in the activity levels of SOD and GSH-Px, in addition to an increase in the level of MDA following treatment with 400 μM H_2O_2. The reduced SOD and GSH-Px activity was attenuated following pretreatment with luteolin, with 25 and 50 μg/ml luteolin significantly ameliorating the increased MDA levels following H_2O_2 treatment (Fig. 3).

Effect of luteolin on the Bcl-2/Bax ratio in PC12 cells. To further investigate the effect of luteolin on H_2O_2-induced PC12 cell apoptosis, the Bcl-2/Bax ratio was measured. The western blotting results demonstrated that the Bcl-2/Bax ratio was reduced in PC12 cells in the H_2O_2-treated group compared with the control group (Fig. 4). However, pretreatment with luteolin significantly attenuated this reduction.

Effect of luteolin on the PI3K/Akt pathway in PC12 cells. The western blot analysis demonstrated that the luteolin treatment significantly increased the levels of p-Akt (Fig 5A). To investigate whether the protective effects of luteolin were mediated through the PI3K/Akt pathway, PC12 cells were pretreated with LY294002, a PI3K/Akt inhibitor. The results demonstrated that the effects of luteolin on p-Akt levels (Fig. 5A), cell viability (Fig. 5B) and the Bcl-2/Bax ratio (Fig. 5C) were reduced following the pretreatment with LY294002.

Discussion

Previous studies have demonstrated that oxidative stress is important in the activation of apoptosis and neuronal cell death in neurodegenerative diseases (24-26). H_2O_2 generates superoxide and hydroxyl radicals, the major components of ROS, and has been extensively used to induce oxidative stress *in vitro* (8). PC12 cells are commonly used

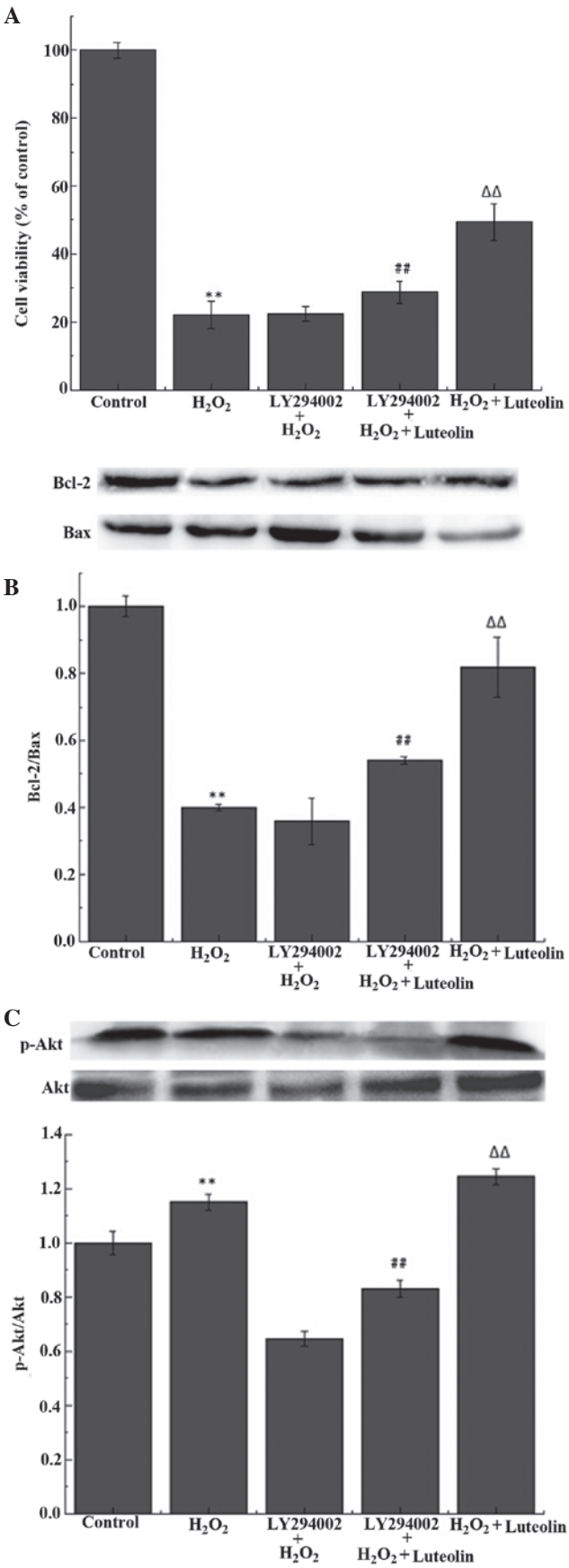

Figure 5. Involvement of phosphoinositide 3-kinase/Akt in the protective effect of luteolin on apoptosis induced by H_2O_2 in PC12 cells. A luteolin concentration of 50 μg/ml was used. (A) Level of p-Akt, (B) cell viability, (C) Bcl-2/Bax ratio. $^{**}P<0.01$ vs. control group, $^{\Delta\Delta}P<0.01$ vs. H_2O_2 treated alone group, $^{\#\#}P<0.01$ vs. H_2O_2 + luteolin group. Akt, protein kinase B; p-Akt, phosphorylated Akt; PC12, rat pheochromocytoma cells.

for neurobiological and neurochemical studies (12,27,28). Therefore, in the current study H_2O_2-induced cytotoxicity was investigated in PC12 cells. Luteolin has been demonstrated to exhibit anti-inflammatory, anti-oxidative and anti-carcinogenic effects (16-18). The current study investigated whether luteolin has protective effects against H_2O_2-induced apoptosis in PC12 cells, and therefore whether it may be of clinical importance.

LDH is an enzyme involved in glycolysis, and cell damage results in the release of LDH, therefore the activity levels of LDH are used as an indicator of cellular integrity. ROS are a product of the aerobic metabolism, and the excess generation of ROS results in lipid peroxidation (29). Cells possess endogenous antioxidants such as GSH-Px and SOD, which scavenge ROS to prevent cell damage. The predominant physiological functions of GSH-Px are free radical scavenging, antioxidant activity and anti-aging activity (30). SOD is able to transform intracellular superoxide anions into H_2O_2. MDA is the end-product of oxygen-derived free radicals and lipid oxidation, and may be used as an indicator of oxidative damage (31). The current study demonstrated that luteolin was able to inhibit the reduction in cell viability induced by H_2O_2. In addition, luteolin was able to reduce ROS formation and LDH release in H_2O_2-treated PC12 cells. SOD and GSH-Px activity were observed to increase following treatment with luteolin, while MDA was reduced. Together, this demonstrates that luteolin was able to increase antioxidant defense, reduce the production of ROS and cellular damage, indicating that luteolin has protective effects against H_2O_2-induced damage in PC12 cells.

The Bcl-2 and Bax genes have been demonstrated to serve a key role in determining whether a cell survives or undergoes apoptosis (32). Bcl-2 and Bax are Bcl-2 family members, and Bcl-2 is involved in the maintenance of cell survival, while Bax serves to accelerate apoptosis. Bcl-2 and Bax have been suggested to be implicated in apoptosis induced by ROS-generating agents (33). In the current study, following pretreatment with luteolin the expression of Bcl-2 was increased, while the expression of Bax was reduced. These alterations resulted in an increase in the Bcl-2/Bax ratio, which indicates that apoptosis was inhibited. These results indicated that luteolin was able to attenuate H_2O_2-induced apoptosis in PC12 cells.

Akt is a central node in cell signaling downstream of growth factors, cytokines and additional cellular stimuli. It promotes cell survival and protects against apoptosis through its ability to phosphorylate and inactivate apoptotic factors (34). Previous studies have indicated that in response to oxidants such as H_2O_2, Akt was rapidly activated (35,36). Futhermore, a previous study demonstrated that Bcl-2 acts downstream of the PI3K/Akt signaling pathway, and that upregulation of Bcl-2 serves an important role in cell survival (37). In the present study, the results demonstrated that luteolin enhanced the PI3K/Akt pathway in response to H_2O_2.

LY294002 is a selective inhibitor of PI3K, which was demonstrated in the current study to attenuate the effect of luteolin on cell viability, Akt phosphorylation and the Bcl-2/Bax ratio. These results suggest that luteolin was able to protect the PC12 cells against H_2O_2-induced apoptosis via reducing ROS levels and activating the PI3K/Akt signaling pathway.

In conclusion, the current study demonstrated that luteolin protected PC12 cells from H_2O_2-induced apoptosis, via the activation of the PI3K/Akt signaling pathway. Therefore luteolin may have protective effects, and further study is required to fully elucidate the protective mechanisms.

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