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Apigenin inhibits indoxyl sulfate-induced endoplasmic reticulum stress and anti-proliferative pathways, CHOP and IL-6/p21, in human renal proximal tubular cells

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Abstract. – OBJECTIVE: Indoxyl sulfate (IS) has been reported to induce endoplasmic reticulum (ER) stress in tubular cells and to inhibit the cell proliferation via ER stress and ERK/IL-6/p21 pathways. This study has investigated the effect of apigenin on IS-induced ER stress in immortalized human renal proximal tubular HK-2 cells.

MATERIALS AND METHODS: Human Kidney 2 (HK-2) cells were treated with IS (5 mM) in the absence or presence of apigenin (10 μM) or salubrinal (20 μM) for indicated times under the serum-free condition. Cell viability was evaluated by MTT assay. The levels of protein expression and phosphorylation were evaluated by Western blot analysis.

RESULTS: In HK-2 cells, apigenin completely inhibited IS-induced ER stress, as indicated by decreased expression of CHOP, ATF4 and GRP78, although the phosphorylated level of eIF2α did not decrease. IS-induced expression levels of IL-6 and p21 proteins were also inhibited by apigenin, with no significant changes in ERK activation. The suppression of cell proliferation by IS was abolished by salubrinal, an ER stress inhibitor, but not by apigenin. Apigenin inhibited the phosphorylation of Akt and GSK-3β in IS-treated HK-2 cells. The phosphorylation of GSK-3β, which was inhibited by apigenin, resulted in hypo-phosphorylation of retinoblastoma (Rb) protein, which was associated with the decrease in cyclin D1 expression.

CONCLUSIONS: These results suggest that apigenin may inhibit IS-induced ER stress and expression of IL-6 and p21 proteins in HK-2 cells. It is most likely that apigenin, together with its inhibitory effect on ER stress, may also suppress the cell growth by inducing the loss of Rb phosphorylation, which was associated with the decrease in cyclin D1 expression by GSK-3β activation through the inhibition of PI3K/Akt pathway.

Key Words:
Apigenin, CHOP, ER stress, HK-2 cells, Indoxyl sulfate, p21.

Introduction

The endoplasmic reticulum (ER) is the intracellular organelle responsible for several cellular functions including the regulation of synthesis, folding, posttranslational modification, and delivery of biologically active proteins[1,2]. The accumulation of unfolded proteins in the lumen of the ER causes ER stress and induces a coordinated adaptive program called the unfolded protein response (UPR). The primary purpose of UPR is adaptation to environmental changes and returns to normal ER functions. However, the UPR also results in cell death when ER stress is severe and prolonged[2,3].

Indoxyl sulfate (IS), a uremic toxin, is metabolized by the liver from indole, which is generated from tryptophan in dietary proteins by intestinal flora including *Escherichia coli* in the large intestine. IS is normally excreted into urine, but is accumulated in serum and the renal tubules when renal function deteriorates[4-7]. IS stimulates the progression of chronic kidney disease (CKD)[5-9]. In CKD patients, IS also shows several deleterious effects such as dys-

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function of cardiac fibroblasts, myocytes, endothelial cells, vascular smooth muscle cells, aortic calcification and atherosclerosis[10-16]. Kawakami et al[17] have reported that IS inhibits proliferation of human renal proximal tubular cells *via* ER stress. IS induced ER stress in tubular cells and inhibited cell proliferation *via* two pathways downstream of ER stress, namely CHOP and ERK/IL-6/p21. Knockdown of CHOP expression by small interference RNAs (siRNAs) significantly mitigated suppression of cell growth in IS-treated tubular cells. Also, IS induced the cell cycle regulator p21 through IL-6 expression in tubular cells, and the increased level of p21, in turn, inhibited cell proliferation. ATF4, a key UPR component, is involved in the up-regulation of IL-6 by IS. Knockdown of ATF-4 by siRNAs significantly attenuated IL-6 induction by IS in tubular cells. These findings suggest that suppression of ER stress is a potential therapeutic target to reduce CKD progression.

Apigenin is a member of the flavone subclass of flavonoids abundantly present in fruits and vegetables, such as onions, oranges, parsley, and chamomile[18,19]. Apigenin has been reported to have various biological activities, such as antioxidant, anti-inflammatory, anti-mutagenic, and anti-tumorigenic properties in various cell types[20-23]. Choi et al[24] have recently reported that apigenin protects HT22 murine hippocampal neuronal cells against ER stress-induced apoptosis. In HT22 cells, apigenin reduced thapsigargin- and brefeldin A-induced ER stress by decreasing the expression of CHOP, GRP78 and GRP94, the cleavage of ATF6α, and the phosphorylation of eIF2α and IRE1α, thereby, inhibiting ER stress-induced apoptosis. In the light of such an inhibitory effect of apigenin against ER stress, it would be of interest to investigate whether apigenin could also reduce IS-induced ER stress in tubular cells. In the present study, we demonstrate that apigenin is capable of reducing IS-induced ER stress in human renal proximal tubular HK-2 cells, with no significant influence on cell proliferation.

Materials and Methods

Reagents and Antibodies

Apigenin (4',5,7-trihydroxyflavone), indoxyl sulfate (IS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and protease inhibitor cocktail were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F12, trypsin-EDTA, fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from GIBCO (Grand Island, NY, USA). Anti-ATF4, anti-β-actin, anti-CHOP, anti-cyclin D1, anti-GRP78 and anti-p21 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Akt, anti-phospho-Akt (Ser473), anti-eIF2α, anti-phospho-eIF2α (Ser51), anti-ERK, anti-phospho-ERK, anti-GSK-3β, anti-phospho-GSK-3β (Ser9), anti-Rb, and anti-phospho-Rb (Ser780) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-human IL-6 antibody was purchased from R&D Systems (Minneapolis, MN, USA). HRP-conjugated goat anti-rabbit IgG, rabbit anti-mouse IgG and rabbit anti-rat IgG antibodies were purchased from Invitrogen (Burlington, ON, Canada).

Cell Culture and Treatment

Immortalized human renal proximal tubular (HK-2) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM/F12 (1:1) supplemented with 10% FBS in 1:100 dilution of an antibiotic-antimycotic solution at 37ºC in a 5% CO_2 incubator. Exponentially growing cells were seeded into a culture dish at 1×10^5 cells/ml and preincubated in complete medium for 24 h. After preincubation, cells were treated with IS (5 mM) in the absence or presence of apigenin (10 μM) for indicated times under the serum-free condition.

MTT Assay

Cell viability was determined using the MTT assay. HK-2 cells were seeded into a 12 well plate at 5×10^4 cells/well. The cells were treated with IS in the absence or presence of apigenin for 24 h under the serum-free condition. After treatment, the cells were washed with serum-free medium, and 1 ml of MTT solution (0.5 mg/ml in serum-free medium) was added to each wells. After incubation for 4 h at 37ºC, MTT containing medium was then removed by aspiration. The blue formazan product generated was dissolved by the addition of 500 μl of 100% dimethyl sulfoxide (DMSO) per well. The amount of formazan was determined at 570 nm using SpectraMAX 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The percent of cell proliferation was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

Western Blot Analysis

Cells were washed with ice-cold phosphate buffered saline (PBS): (pH 7.4) and gently resuspended in ice-cold RIPA buffer with freshly added 1% protease inhibitor cocktail, incubated on ice for 30 min. Cell lysates were centrifuged at 14,000 rpm for 15 min at 4ºC, and the protein concentration was determined using a Bradford assay. Samples containing 50 µg of total protein were resolved by SDS-PAGE gel, and transferred onto a nitrocellulose membrane for 3 h at 40V. The membranes were blocked with Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk and probed with primary antibodies (all 1:1000 in 3% BSA in Tris-buffered saline with Tween-20) overnight at 4ºC with gentle shaking. Protein spots were detected using HRP-conjugated secondary antibodies (all 1:2000 in 3% BSA in Tris-buffered saline with Tween-20). Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Waltham, MA, USA) and then developed using the FluorChem E System (ProteinSimple, San Jose, CA, USA). The density of bands was quantitated using Quantity One 4.6.6 software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was performed using Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA). The data were expressed as means ± standard deviation (SD). The statistically significant differences between two groups were calculated by Student's *t*-test. p-value < 0.05 was considered to indicate statistically-significant differences.

Results

Effect of Apigenin on IS-induced ER Stress

It has been reported that IS can induce ER stress in renal tubular cells[17]. We examined whether apigenin could inhibit IS-induced ER stress in HK-2 cells. The cells were treated with IS in the absence or presence of apigenin in serum-free medium, and the expression and phosphorylation levels of ER stress-related proteins, then, were determined by Western blot analysis. Expression of CHOP, ATF4 and GRP78, and phosphorylation of eIF2α were time-dependently increased when the cells were treated with IS alone (Figure 1). Apigenin almost completely blocked IS-induced expression of CHOP, ATF4 and GRP78 (Figure 1A), whereas it did not block the phosphorylation of eIF2α (Figure 1B). Surprisingly, treatment with apigenin resulted in a further increase in the levels of phosphorylated eIF2α.

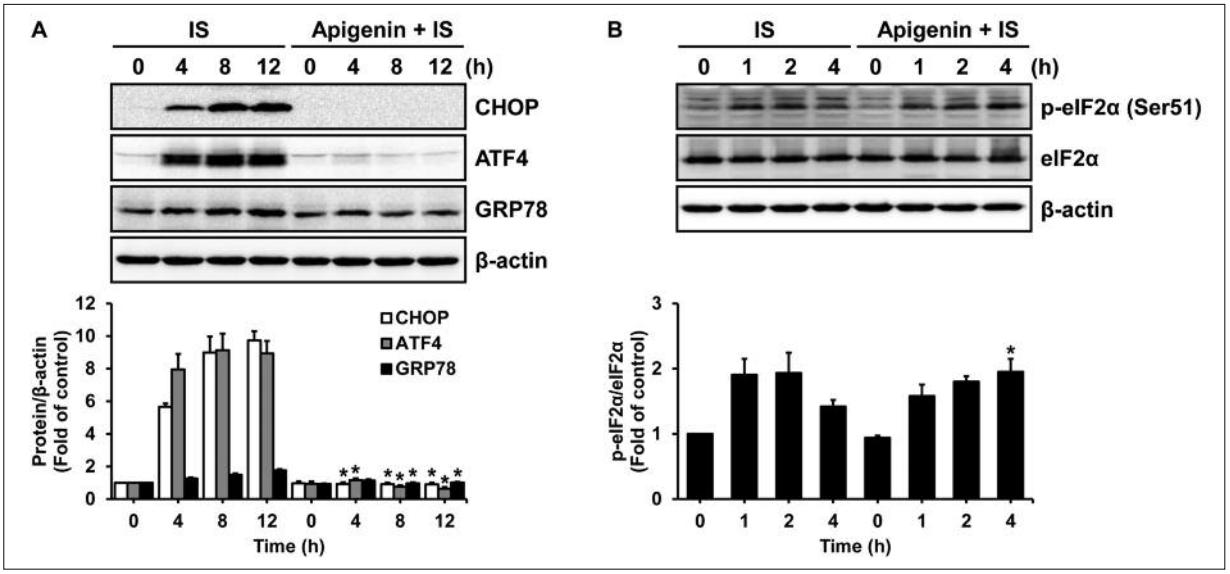

Figure 1. Cells were treated with 5 mM IS in the absence or presence of 10 µM apigenin for indicated times in serum-free medium. Cell lysates were subjected to Western blot analysis with ***(A)*** anti-CHOP, anti-ATF4, anti-GRG78, ***(B)*** phospho-anti-eIF2α and anti-eIF2α antibodies. The density of band was quantitated by densitometry using Bio-Rad Quantity One software. Value are means ± SD, N = 3. *p < 0.05 vs. IS-alone treated group.

Effects of Apigenin on IS-induced IL-6 and p21 Expression

IS-induced ER stress on renal tubular cells has been shown to activate IL-6/p21 pathway *via* ERK phosphorylation, which is suggested to be involved in IS-induced inhibition of cell proliferation[17]. We, thus, investigated whether apigenin could inhibit the ERK/IL-6/p21 pathway in IS-treated HK-2 cells. The cells were treated with IS in the absence or presence of apigenin, and then phosphorylation of ERK and expression of IL-6 and p21 were determined by Western blot analysis. As shown in Figure 2, apigenin inhibited IS-induced expression of IL-6 and p21, with no significant change in the phosphorylation level of ERK. It should be noted that apigenin itself could induce ERK phosphorylation (Figure 2A).

The p21 is well known as one of potent cyclin-dependent kinase inhibitors that are involved in regulation of cell cycle progression and cell cycle arrest[25]. Accordingly, we speculated that in IS-treated cells, the loss of p21 expression by apigenin could result in elevation of cell proliferation. To test this hypothesis, HK-2 cells were treated with IS in the absence or presence of apigenin or salubrinal, an ER stress inhibitor, and then cell proliferation was determined by MTT assay. As shown in Figure 3, apigenin did not prevent the suppression of cell proliferation by IS, but rather decreased the cell proliferation than that of the cells treated with IS alone. In contrast, treatment with salubrinal resulted in a significant increase in cell proliferation, as compared to IS-treated cells. It should be noted that apigenin itself was antiproliferative (Figure 3).

Effect of Apigenin on IS-induced Akt and GSK-3β Activation

Apigenin is a potent inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt pathway[26]. The PI3K/Akt pathway is known to play a major role in cell cycle progression[27]. One of the major effectors downstream of PI3K/Akt pathway is glycogen synthase kinase (GSK)-3. Upon the activation of Akt, Akt inactivates GSK-3β by phosphorylating it at serine 9 residue[28]. GSK-3β negatively regulates cell cycle progression[28,29]. We examined whether IS treatment could induce Akt and GSK-3β phosphorylation in HK-2 cells, and if so, whether apigenin could inhibit IS-induced Akt and GSK-3β phosphorylation. The cells were treated with IS in the absence or presence of apigenin in serum-free medium, and then phosphorylation levels of Akt and GSK-3β were determined by Western blot analysis. As shown in Figure 4, IS increased the phosphorylation levels of Akt and GSK-3β, and apigenin significantly reduced these levels, as compared to the cells treated with IS alone. It should be noted that the phosphorylation levels of Akt and GSK-3β in the

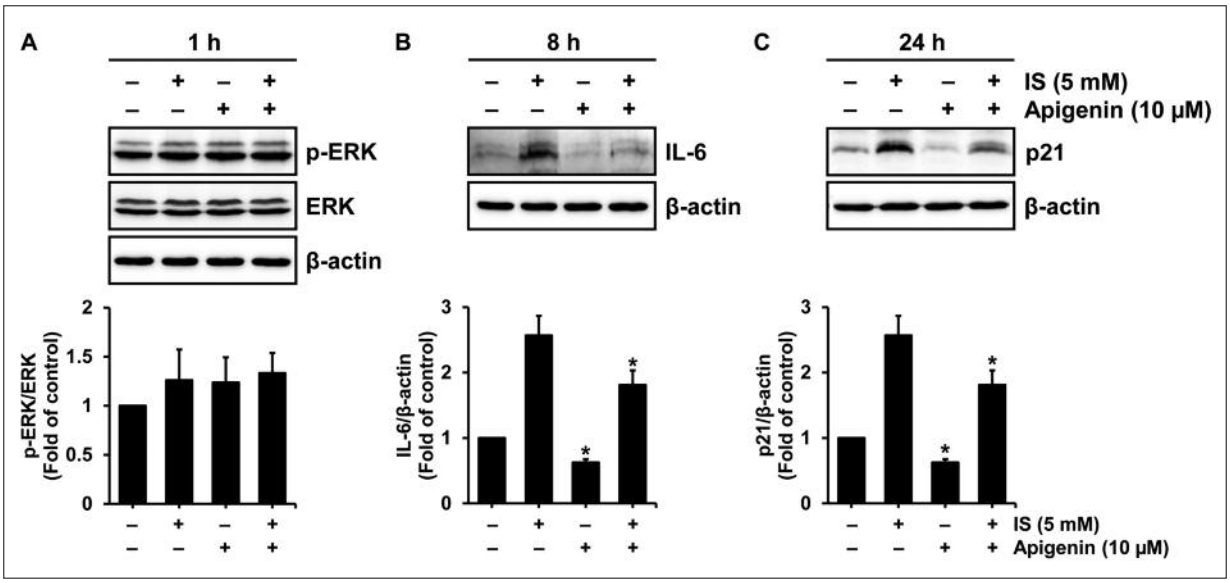

Figure 2. Cells were treated with 5 mM IS in the absence or presence of 10 μM apigenin for indicated times in serum-free medium. Cell lysates were subjected to Western blot analysis with ***(A)*** anti-phospho-ERK, anti-ERK, ***(B)*** anti-IL6 and ***(C)*** anti-21 antibodies. The density of band was quantitated by densitometry using Bio-Rad Quantity One software. Value are means ± SD, N = 3. *$p < 0.05$ vs. IS-alone treated group.

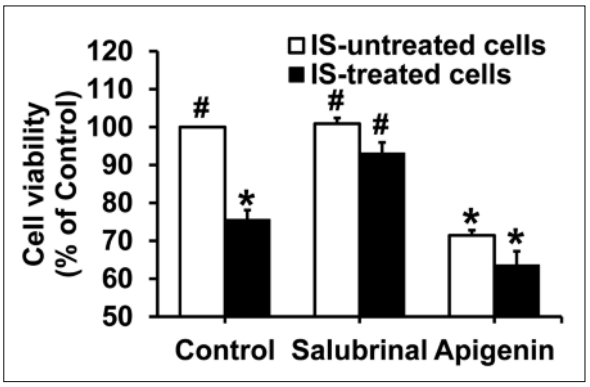

Figure 3. Cells were treated with 5 mM IS in the absence or presence of 10 μM apigenin or 20 μM salubrinal for 24 h in serum-free medium. Cell viability was measured by MTT assay. Values are means ± SD, N = 3. *$p < 0.05$ vs. untreated control group. #$p < 0.05$ vs. IS-alone treated group.

cells treated with apigenin alone were significantly lower than their basal phosphorylation levels in untreated control cells.

Effects of Apigenin on cyclin D1 Expression and Rb Phosphorylation

GSK-3β phosphorylates the cyclin D1 at Thr 286 residue that triggers its subsequent ubiquitination and proteasomal degradation[29,30]. Cyclin D1 is an essential cell cycle regulatory molecule, which allows G1/S phase entry and cell division by binding to cyclin-dependent kinase (CDK) 4. Cyclin D1-CDK4 phosphorylates the retinoblastoma (Rb) protein at Ser 780 residue, upon which E2F is released to transactivate the genes required for the G1- to S-phase progression[28,31,32]. We examined whether apigenin could inhibit cyclin D1 expression and Rb phosphorylation in HK-2 cells. The cells were treated with IS in the absence or presence of apigenin in serum-free medium, and then cyclin D1 expression and Rb phosphorylation were determined by Western blot analysis. As shown in Figure 5, IS alone had no significant effect on cyclin D1 expression and Rb phosphorylation, whereas apigenin reduced cyclin D1 expression and Rb phosphorylation, even in IS-treated cells.

Discussion

The uremic toxin IS induces the ER stress, and inhibits cell proliferation through increased expression of p21 in cultured human renal proximal tubular cells[9]. In the present study, we demonstrate that apigenin inhibits the IS-induced ER stress and p21 expression in HK-2 cells. Despite its ability to inhibit IS-induced ER stress and p21 expression, apigenin could not abolish antiproliferative effect of IS.

The ER stress can lead to either cell growth suppression or cell death[33-35]. It has been reported that IS-induced ER stress inhibits proliferation of human renal proximal tubular cells *via* CHOP and ERK/IL-6/p21 pathways[17]. Our results also show that IS induced the ER stress responses, including up-regulation of CHOP, ATF4, GRP78 and phospho-eIF2α expressions, and suppressed the proliferation in HK-2 cells. Interestingly, apigenin, an edible plant-derived flavonoid, was found to reduce IS-induced ER stress; it almost completely blocked IS-induced expression of CHOP, ATF4 and GRP78, with no significant

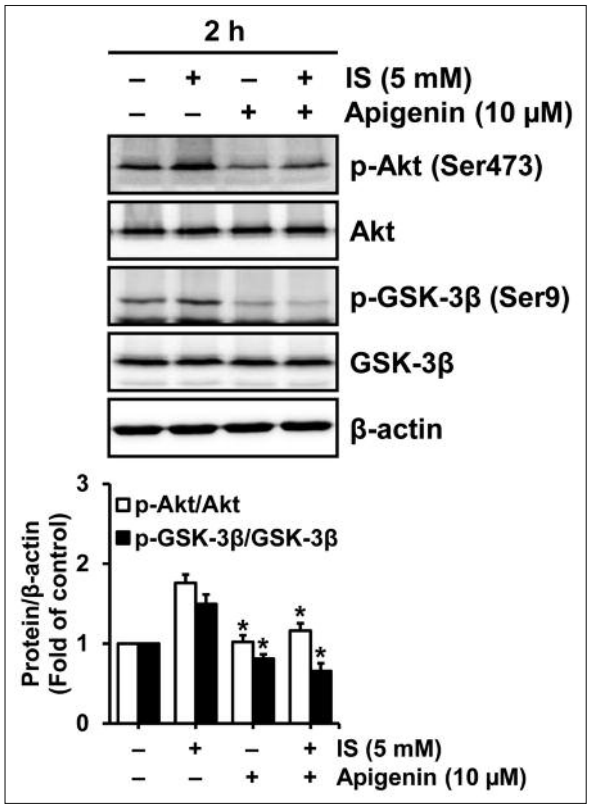

Figure 4. Effects of apigenin on PI3K/Akt/GSK-3 pathway in IS-treated HK-2 cells. Cells were treated with 5 mM IS in the absence or presence of 10 μM apigenin for 2 h in serum-free medium. Cell lysates were subjected to Western blot analysis with anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-GSK-3β (Ser9) and anti-GSK-3β antibodies. The density of band was quantitated by densitometry using Bio-Rad Quantity One software. Value are means ± SD, N = 3. *$p < 0.05$ vs. IS-alone treated group.

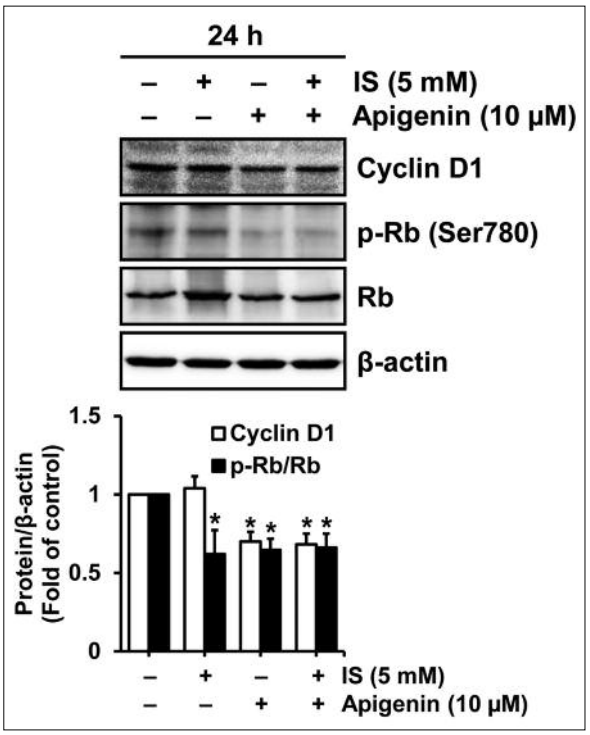

Figure 5. Effects of apigenin on expression of cyclin D1 and Rb, and phosphorylation of Rb in IS-treated HK-2 cells. Cells were treated with 5 mM IS in the absence or presence of 10 μM apigenin for 24 h in serum-free medium. Cell lysates were subjected to Western blot analysis with anti-cyclin D1, anti-phospho-Rb (Ser780) and anti-Rb antibodies. The density of band was quantitated by densitometry using Bio-Rad Quantity One software. Value are means ± SD, N = 3. *$p < 0.05$ vs. untreated control group.

change in eIF2α phosphorylation. Furthermore, apigenin also inhibited IS-induced IL-6/p21 pathway, with no significant change in ERK activation. Apigenin has been shown to inhibit the production of pro-inflammatory mediators, such as IL-6, in several cell lines[36-38]. Thus, it is most likely that the reduction of p21 expression by apigenin in IS-treated HK-2 cells may be related, at least in part, to its ability to down-regulate IL-6 expression. Surprisingly, apigenin did not prevent IS-induced suppression of cell proliferation despite its inhibition of ER stress and IL-6/p21 pathways. This effect is probably due to an antiproliferative effect of apigenin, because the ER stress inhibitor salubrinal prevented IS-induced suppression of cell proliferation.

Apigenin has been reported to suppress cell proliferation in various cell types, and such an anti-proliferative effect has been shown to be associated with PI3K/Akt pathway[27,39]. The PI3K/Akt pathway is one of signal transduction pathways activated by several membrane receptors, including growth factor and G protein-coupled receptors, and plays an important role in cell cycle progression[27,40]. Among several target proteins of Akt, GSK-3β is one of serine/threonine protein kinases that are involved in cell cycle regulation[41]. Upon activation, GSK-3 phosphorylates cyclin D1 on Thr286, which promotes its degradation by subsequent proteasomal ubiquitination[29,30,41]. The PI3K/Akt pathway is capable of phosphorylating GSK-3β at Ser9 and subsequently inhibiting its kinase activity[28]. In contrast, inactivation of PI3K/Akt pathway will activate GSK-3β, which results in a decrease in cyclin D1 levels. Apigenin is known as a potent inhibitor of PI3K/Akt pathway[26]. This study demonstrated that apigenin inhibited Akt phosphorylation in IS-treated HK-2 cells. Inhibition of Akt phosphorylation by apigenin resulted in decreased expression of cyclin D1 as well as phosphorylation of GSK-3β. Finally, apigenin-mediated down-regulation of cyclin D1 led to reduction of Rb phosphorylation at Ser780. Rb, a tumor suppressor protein that regulates the cell cycle progression through cooperating with cyclins and CDKs. Phosphorylation of Rb by CDKs/cyclins complex inhibits Rb-E2F binding to allow E2F activation and cell cycle progression[42]. Among the phosphorylation sites of Rb, cyclin D1 is required for Ser780 phosphorylation of Rb[31]. Thus, it is most likely that antiproliferative effect of apigenin observed in this study may be due to hypo-phosphorylation of Rb *via* its inhibition of PI3K/Akt pathway.

Meanwhile, GSK-3β activity has been reported to be influenced by ER stress-responsive eIF2α[43,44]. The eIF2α is capable of promoting the nuclear localization of GSK-3β concomitantly with an induction of its activity[44]. In our study, IS induced the activation of eIF2α in HK-2 cells, and apigenin did not inhibit IS-activated eIF2α; instead, apigenin significantly increased the phosphorylation levels of eIF2α when the cells were treated with IS in the presence of apigenin. Consequently, the activation of eIF2α by apigenin is speculated to enhance its antiproliferative effect in IS-treated HK-2 cells.

Conclusions

Apigenin reduces ER stress triggered by the uremic toxin IS in HK-2 cells, but does not abolish the antiproliferative effect of IS. In ad-

dition to its inhibition of ER stress, apigenin may have other ability to induce the hypo-phosphorylation of Rb, which was associated with a significant decrease in cyclin D1 expression by GSK-3β activation through inhibition of PI3K/Akt pathway and also with a significant increase in activation of eIF2α kinase. This may explain why apigenin could not abolish the antiproliferative effect of IS. These findings suggest the possibility of a new therapeutic approach based on apigenin compounds that can prevent CKD progression associated with IS accumulation in kidney.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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