Original Article

DOI: 10.5582/bst.2017.01218

Induction of apoptosis by ethanol extract of *Citrus unshiu* Markovich peel in human bladder cancer T24 cells through ROS-mediated inactivation of the PI3K/Akt pathway

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Summary ***Citrus unshiu*** **peel has been used to prevent and treat various diseases in traditional East-Asian medicine including in Korea. Extracts of *C. unshiu* peel are known to have various pharmacological effects including antioxidant, anti-inflammatory, and antibacterial properties. Although the possibility of their anti-cancer activity has recently been reported, the exact mechanisms in human cancer cells have not been sufficiently studied. In this study, the inhibitory effect of ethanol extract of *C. unshiu* peel (EECU) on the growth of human bladder cancer T24 cells was evaluated and the underlying mechanism was investigated. The present study demonstrated that the suppression of T24 cell viability by EECU is associated with apoptosis induction. EECU-induced apoptosis was found to correlate with an activation of caspase-8, -9, and -3 in concomitance with a decrease in the expression of the inhibitor of apoptosis family of proteins and an increase in the Bax:Bcl-2 ratio accompanied by the proteolytic degradation of poly(ADP-ribose) polymerase. EECU also increased the generation of reactive oxygen species (ROS), collapse of mitochondrial membrane potential, and cytochrome c release to the cytosol, along with a truncation of Bid. In addition, EECU inactivated phosphatidylinositol 3-kinase (PI3K) as well as Akt, a downstream molecular target of PI3K, and LY294002, a specific PI3K inhibitor significantly enhanced EECU-induced apoptosis and cell viability reduction. However, N-acetyl cysteine, a general ROS scavenger, completely reversed the EECU-induced dephosphorylation of PI3K and Akt, as well as cell apoptosis. Taken together, these findings suggest that EECU inhibits T24 cell proliferation by activating intrinsic and extrinsic apoptosis pathways through a ROS-mediated inactivation of the PI3K/Akt pathway.**

Keywords: *Citrus unshiu* peel, apoptosis, PI3K/Akt, ROS

Released online in J-STAGE as advance publication October 24, 2017.

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1. Introduction

The progression of cancer is a multistep process involving genetic modification, and the deregulation or mutation of particular genes is likely to be a marker for certain cancers. Bladder cancer has been known as one of the most frequent urological malignancies with a high incidence and mortality rate (*1,2*). Although recent advances in treatment have led to increased survival rates for patients with bladder cancer, the incidence

and mortality rates are still increasing (*3,4*). Therefore, it is imperative to understand the basic mechanisms of bladder cancer progression and to find new biological targets and effective treatment strategies for bladder cancer prevention and treatment.

Recently, several modes of programmed cell death associated with the inhibition of the proliferation of cancer cells by chemotherapeutic drugs have been described (*5,6*). Among them, apoptosis, the most typical cell death mechanism, can be triggered through either a death receptor (DR)-initiated extrinsic pathway or a mitochondria-mediated intrinsic pathway characterized by the activation of common caspases (*7,8*). The extrinsic pathway triggers apoptosis through the binding of death ligands to the DRs, which activates the caspase cascade from the upstream initiator caspase-8 to the downstream effector caspases, including caspase-3 and -7, by recruiting adapter molecules (*9,10*). The intrinsic pathway is mainly regulated by the interaction between the Bcl-2 family of proteins composed of proteins capable of promoting or inhibiting apoptosis, which is also associated with impaired mitochondrial function. This in turn promotes the release of apoptotic factors such as cytochrome *c* from the mitochondria to the cytoplasm, activating caspase-9, ultimately leading to a caspase cascade pathway that activates the effector caspases (*7,11*). Although reactive oxygen species (ROS) act as major regulators of cell survival and proliferation, excessive production of ROS causes irreversible cellular damage in various cell death types (*12,13*). In addition, the induction of cancer cell death associated with mitochondrial dysfunction in the intrinsic apoptosis pathway is often connected with the overproduction of ROS (*14,15*).

Such apoptosis induction is complexly regulated by the activation and inactivation of various intracellular signal pathways in the cell. Among them, the phosphatidylinositol 3-kinase (PI3K) is abnormally activated or mutated in many tumors, including bladder cancer, and its activation is one of the most important tumorigenic pathways in cancer (*16,17*). PI3K induces the activation of Akt, a downstream effector of PI3K, through the phosphorylation of major amino acid residues such as Thr 308 and Ser 473 to stimulate cancer proliferation (*18,19*). Therefore, many clinical trials are urgently needed to find new agents that interfere with the signaling of PI3K pathway components. Interestingly, many previous studies show that increased production of ROS and inactivation of the PI3K/Akt signal pathway are related to the induction of cancer cell apoptosis (*20-22*). Although studies on the role of ROS production in PI3K/Akt inactivation have not been fully understood, ROS-dependent PI3K/Akt signaling pathway blockade may be a potential therapeutic target for inducing apoptosis in cancer cells (*23,24*).

Recently, the discovery of cancer substances using natural compounds, especially plant-derived compounds, has received considerable interest. *Citrus unshiu* Markovich, which belongs to the Rutaceae family, is a citrus fruit that is readily seeded, has no seeds, and is grown in East-Asian countries, including Korea (*25,26*). For several hundred years, citrus and dried peel have been used as traditional medicines to treat gastrointestinal disorders, colds, indigestion, and bronchial discomfort, and pharmacological activities have been reported for inflammation, allergies, diabetes, and viral infections (*27-30*). A study on a tumor-bearing mouse model has shown that *C. unshiu* peel (Chimpi) extract inhibits tumor growth, which was associated with an increased production of cytokines such as interferon-γ and tumor necrosis factor-α (*31*). In addition, Kim *et al*. (*32*) reported that *C. unshiu* peel reduces systemic inflammation in tumor-bearing mice and inhibits the production of pro-cacheche factors in tumors with the prevention of skeletal muscle atrophy and weight loss. It has also been reported that polysaccharides or flavonoids found in the *C. unshiu* peel can inhibit the metastasis of cancer cells (*33,34*); however, the evidence for the therapeutic potentials of *C. unshiu* peel against human cancer cells and the molecular pathways involved in suppression of cancer cell apoptosis remain unclear. Therefore, we investigated the anti-cancer effects of ethanol extract of *C. unshiu* peel (EECU) in human bladder cancer T24 cells. In this study, we found for the first time that EECU triggered apoptotic cell death through the ROS-mediated inactivation of the PI3K/Akt pathway.

2. Materials and Methods

2.1. *Preparation of EECU*

For the preparation of EEER, the dried peel of *C. unshiu* was provided from Dongeui Korean Medical Center (Busan, Republic of Korea) and pulverized into a fine powder. The powder (100 g) was extracted in 1 L of 70% ethanol by sonication for 24 h. After filtering, the filtrate was concentrated with a vacuum rotary evaporator (BUCHI, Switzerland) and the residue was freeze-dried in a freezing-dryer, and then stored at –80°C. The powder was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) to a final concentration of 100 mg/mL (extract stock solution), and was stored at 4°C. The stock solution was diluted with medium to the desired concentrations prior to use.

2.2. *Cell culture*

Human urinary bladder transitional cell carcinoma T24 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in RPMI 1640 medium (WelGENE Inc., Daegu, Republic of Korea), supplemented with 10%

fetal bovine serum (FBS, WelGENE Inc.), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (WelGENE Inc.) at 37°C in a humidified incubator containing 5% CO_2.

2.3. *Cell viability assay and morphological observation*

To assess the effects of EECU on T24 cell viability, the cells were plated at a density of 2×10^4 cells per well in a 24-well plate. After overnight incubation, the cells were treated with different concentrations of EECU for 48 h. Following treatment, the culture medium containing EECU was carefully removed and 200 μL of 0.1 mg/mL 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) solution were added to each well for 2 h at 37°C, prior to dissolving the formazan product using DMSO. The viability of the cells was measured by absorption at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Silicon Valley, CA, USA). The mean percentages of viable cells ± standard deviation (SD) generated from three independent experiments were calculated. The cells in each well were observed under an inverted microscope (Carl Zeiss, Oberkochen, Germany) and were then photographed.

2.4. *Colony formation assay*

After treatment with different concentrations of EECU for 48 h, single-cell suspensions were prepared by trypsinization and the cells were then seeded into 6-well plates (500 cells/well). The cells were further cultured for two weeks to allow the formation of colonies. The colonies were fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.), stained with 0.1% crystal violet solution (Sigma-Aldrich Chemical Co.) for 10 min, washed, and then imaged under an inverted microscope.

2.5. *Detection of apoptotic morphological changes*

Apoptotic cells containing chromatin condensation and nuclear fragmentation in the nuclei were detected by 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) staining. After treatment with EECU, the cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed with 3.7% paraformaldehyde in PBS for 10 min at 25°C. The cells were washed with PBS, stained with 1 mg/mL of DAPI solution for 10 min, and then washed twice with PBS. The morphology changes in the nucleus were examined using a fluorescence microscope (Carl Zeiss).

2.6. *Determination of cell apoptosis by flow cytometry*

To determine the magnitude of the apoptosis by EECU, the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used as described previously (*35*). Briefly, both attached and floating cells were collected and then washed twice with ice old PBS (resuspended in 500 μL binding buffer). The cells were stained with FITC-conjugated annexin V and propidium iodide (PI) at room temperature for 15 min in the dark. Subsequently, the cells were analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's protocol.

2.7. *Protein isolation and Western blot analysis*

Following incubation for 48 h with different concentrations of EECU, the cells were lysed in a protein extraction buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). For the preparation of mitochondrial and cytosolic extracts of cells, NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific Inc., Waltham, Utah, USA) were applied according to the manufacturer's instructions. Protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. For Western blotting, equal amounts of proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and were transferred onto a polyvinylidene fluoride (PVDF) membrane (Schleicher & Schuell, Keene, NH, USA) using an electrophoretic transfer system (Bio-Rad Laboratories). The membranes were blocked in 5% (w/v) skim milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST buffer) for 1 h at room temperature. After washing with TBST buffer, the membranes were probed with specific primary antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA) at 4°C overnight, and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Life Science, Arlington Heights, IL, USA). The protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Life Science), according to the manufacturer's instructions.

2.8. *Analysis of caspase enzymatic activity*

The activities of the caspases (caspase-3, -8, and -9) were detected using colorimetric assay kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Briefly, the cells were lysed in the supplied lysis buffer. Equal amounts of proteins were incubated with the supplied reaction buffer containing dithiothreitol and synthetic tetrapeptides [Asp-Glu-Val-Asp (DEAD) for caspase-3; Ile-Glu-Thr-Asp (IETD) for caspase-8; and Leu-Glu-His-Asp (LEHD) for caspase-9] labeled with p-nitroaniline

(pNA) that is linked to the end of the caspase-specific substrate at 37°C for 2 h in the dark. The reactions of each sample were measured by changes in absorbance at 405 nm using an ELISA reader.

2.9. *Determination of mitochondrial membrane potential (Δψm)*

The changes of the MMP values in the EECU-treated cells were examined using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma-Aldrich Chemical Co.), a dual-emission potential-sensitive probe. Briefly, the cells were harvested and washed with cold PBS, and incubated with 10 μM JC-1 for 30 min at 37°C in the dark. Then, the stained cells were washed twice with PBS to remove unbound dye, and the amount of JC-1 retained by 10,000 cells per sample was measured at 488 nm and 575 nm using a flow cytometer, following the manufacturer's protocol instructions.

2.10. *Measurement of ROS generation*

For the detection of intracellular ROS production, 2′,7′-dichlorofluorescin diacetate (DCF-DA, Molecular Probes, Leiden, Netherlands) dye was used according to the manufacturer's instructions (*36*). Briefly, after collecting the cells treated with EECU for a certain period of time, the cells were rinsed with PBS and then loaded with 10 μM DCF-DA for 20 min at 37°C in a dark room. The cells were immediately washed, resuspended in PBS, and analyzed to determine the fluorescence intensity using a flow cytometer. To confirm whether the intracellular ROS levels play a role in the cytotoxicity of EECU, the cells were pre-treated with N-acetyl cysteine (NAC, Sigma-Aldrich Chemicals Co.), a well established antioxidant, for 1 h prior to treatment with EECU. The stained cells were also mounted on a chamber slide with a mounting medium. The images were obtained under a fluorescence microscope.

2.11. *Data analysis*

The experimental results were presented as mean ± SD of experiments repeated at least three times. For each treatment group, the statistical significance was compared with that of other groups, and was verified using a one-way ANOVA or Student *t*-test method. A $p < 0.05$ was considered to indicate a statistically significant result.

3. Results

3.1. *EECU inhibits cell growth and colony forming property of T24 cells*

To determine the inhibitory effect of EECU on T24 cell growth, an MTT assay was performed. The obtained results indicated that EECU was shown to inhibit the cell survival rate against T24 cells in a concentration-dependent manner (Figure 1A) and accompanied by various morphological changes including membrane blebbing, diminished cell density, poor adherence, and increased number of floating cells (Figure 1B). In the cologenic assay, EECU also significantly reduced the number of colonies of T24 cells that depended on treatment concentration compared with the control group (Figure 1C), indicating that the ability to form colonies by the EECU-treated T24 cells was lost.

3.2. *EECU induces apoptosis in T24 cells*

To determine whether EECU treatment led to growth reduction due to apoptosis induction, the changes of nucleus morphology by DAPI staining and cell death rate using flow cytometry were investigated. The results of DAPI staining showed that nuclear fragmentation and chromatin condensation found in apoptotic cells increased in the EECU-treated cells compared to the untreated control cells (Figure 1D). In addition, the results from the flow cytometry showed that the

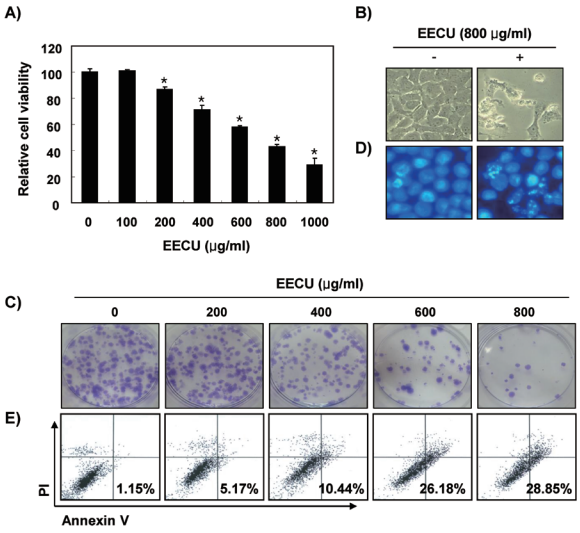

Figure 1. Inhibition of cell viability and induction of apoptosis by EECU in T24 cells. T24 cells were treated with various concentrations of EECU for 48 h. **(A)** The cell viability was measured by an MTT assay. The data were expressed as the mean ± SD of three independent experiments (*$p < 0.05$ *vs*. untreated control). **(B)** The morphological change of MDA-MB-231 cells treated with 800 μg/mL EECU was observed under an inverted microscope (magnification, ×200). **(C)** The cells were exposed to the indicated concentrations of EECU for 48 h and were allowed to form colonies for 14 days. The representative photographs are shown. **(D)** The cells were fixed and stained with DAPI solution. Stained nuclei were then observed with a fluorescence microscope (original magnification, ×400). **(E)** The degree of apoptosis induced by EECU was determined in cells stained with FITC-conjugated Annexin V and PI, and subjected to flow cytometry analysis. Apoptotic cells are determined by counting the % of annexin V^+/PI^- cells. Each point represents the means of two independent experiments.

percentage of apoptotic cells was markedly increased in the EECU treatment groups in a concentration-dependent manner (Figure 1E). These data collectively indicate that EECU suppressed cell viability and colony formation by inducing apoptosis in the T24 cells.

3.3. *EECU activates caspases in T24 cells*

In order to investigate whether the activation of caspases plays a role in EECU-induced T24 cell apoptosis, the levels of caspase-8, -9, and -3 were measured following treatment with various concentrations of EECU. The results revealed that the expression of pro-caspase-8, an initiator caspase of the extrinsic apoptosis pathway, decreased with increasing EECU concentration, while the expression of active-caspase-8 increased (Figure 2A). Although the expression of the active-caspase-9, an initiator caspase of the extrinsic apoptosis pathway, was not detected, the expression of its pro-forms was apparently suppressed depending on the EECU treatment concentration. Our immunoblotting results also revealed a concentration-dependent decrease in caspase-3 pro-form expression, a typical effector caspase, and progressive proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), which is a representative substrate protein of activated effector caspases. Consistent with immunoblotting results, the *in vitro* activity of the three examined caspases was significantly enhanced by EECU treatment (Figure 3B), which was associated with the down-regulation of the inhibitor of apoptosis proteins (IAP) family members such as XIAP, cIAP-1, and cIAP-2 (Figure 2C).

3.4. *EECU modulates the expression of DR-related and Bcl-2 family proteins in T24 cells*

Because the results of Figure 2 show the possibility that two apoptotic pathways may be involved in the induction of apoptosis by EECU, we then evaluated the effect of EECU on the expression of DR-related and Bcl-2 family proteins. The Western blotting data indicated that the expressions of TNF-related apoptosis-inducing ligand (TRAIL), DR4, Fas, and Fas ligand (FasL) increased in response to EECU treatment in a concentration-dependent fashion, even though the expression of DR5 was not changed (Figure 3). In addition, among the Bcl-2 family proteins, the expression of pro-apoptotic Bax was markedly increased when compared with those levels in the control groups, whereas the anti-apoptotic Bcl-2 expression was reduced by EECU treatment. Furthermore, the levels of total Bid expression decreased due to EECU treatment, but truncated Bid (tBid) expression progressively increased depending on the EECU treatment concentration.

3.5. *EECU enhances the disruption of MMP and release of cytochrome c in T24 cells*

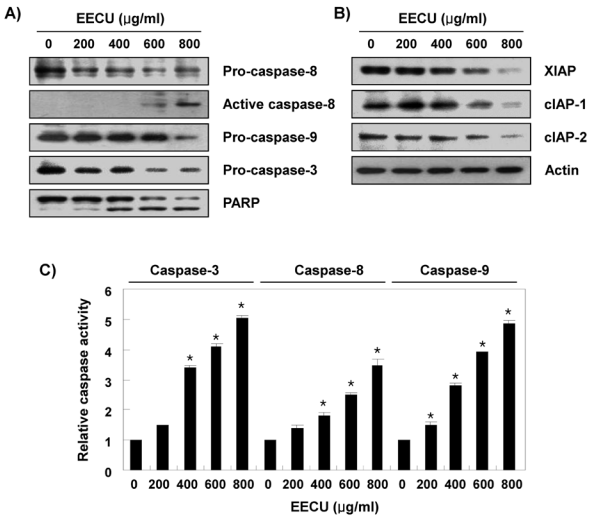

Figure 2. Activation of caspases, degradation of PARP, and inhibition of IAP family proteins expression by EECU in T24 cells. T24 cells were treated with the indicated concentrations of EECU for 48 h. **(A** and **B)** The cell lysates were prepared and equal amounts of cellular proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with the indicated antibodies and the proteins were visualized using an ECL detection system. Actin was used as an internal control. **(C)** The activities of caspases were evaluated using caspases colorimetric assay kits. The data were expressed as the mean ± SD of three independent experiments (*$p < 0.05$ *vs.* untreated control).

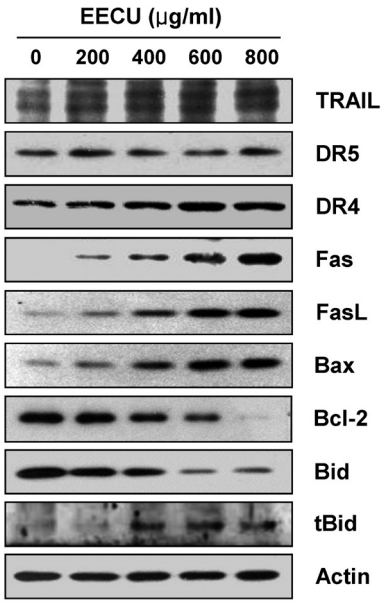

Figure 3. Effects of EECU on the levels of DR-related and Bcl-2 family proteins in T24 cells. After 48 h incubation with the indicated concentrations of EECU, the cells were lysed, and cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Equal protein loading was confirmed by analysis of actin in the protein extracts.

To further investigate whether mitochondrial dysfunction is involved in the induction of apoptosis by EECU, we determined the effects of EECU on MMP values. As shown in Figure 4A, the frequency of cells with JC-1 monomers, which are predominant in the region with low MMP (lower right quadrant of fluorescence cytogram), the concentration-dependency increased in the EECU-treated T24 cells, revealing that EECU markedly destroys the integrity of the mitochondria measured by the concentration-dependent loss of MMP (Figure 4A). Subsequently, an increase of cytochrome *c* protein level in cytoplasm was obviously observed upon treatment

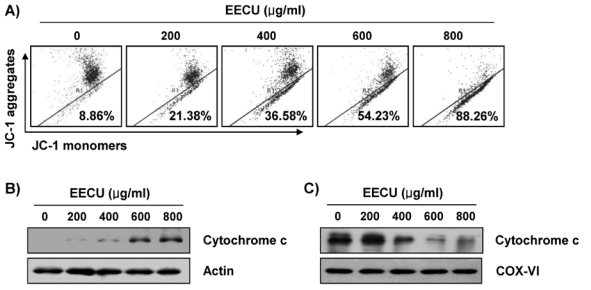

Figure 4. Effects of EECU on the levels of MMP values and cytochrome c expression in T24 cells. (A) After 48 h incubation with the indicated concentrations of EECU, the cells were stained with JC-1 dye and were then analyzed on a flow cytometer in order to evaluate the changes in MMP. The data are expressed as the mean of two independent experiments. **(B** and **C)** Cells cultured under the same conditions were lysed, and cytosolic and mitochondrial proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to the membranes. The membranes were probed with anti-cytochrome c antibody. Proteins were visualized using an ECL detection system. Equal protein loading was confirmed by analysis of actin and cytochrome oxidase subunit VI (COX VI) in each protein extract.

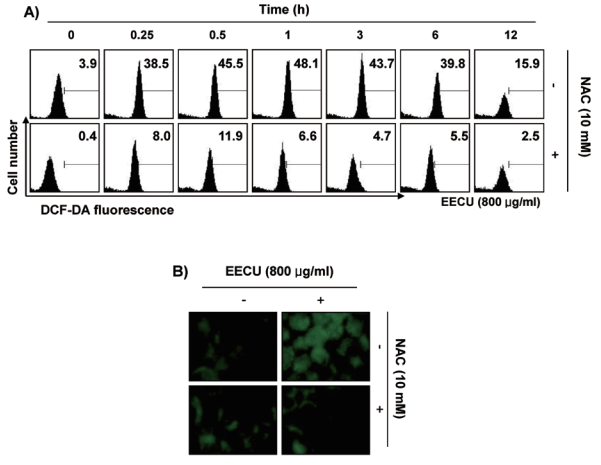

Figure 5. Induction of ROS generation by EECU in T24 cells. (A) T24 cells were either treated with 800 μg/mL EECU for the indicated times or pre-treated with NAC (10 mM) for 1 h before EECU treatment. The medium was discarded and the cells were incubated at 37°C in the dark for 20 min with new culture medium containing 10 μM DCF-DA. ROS generation was measured by a flow cytometer. The data are the means of the two different experiments. **(B)** Images were obtained using a fluorescence microscope (Original magnifications: ×200). The images presented here are captured from one experiment and are representative of at least three independent experiments.

of increased concentrations of EECU (Figures 4B and 4C), suggesting that mitochondrial dysfunction may also contribute to EECU-induced apoptosis in T24 cells.

3.6. *EECU increases the accumulation of ROS in T24 cells*

To assess whether EECU-induced mitochondrial dysfunction is associated with ROS production, we quantified the intracellular ROS levels using a DCF-DA probe. Flow cytometry results in Figure 5A demonstrated that the ROS levels elevated sharply within 25 min after EECU treatment, peaked at 1 h of EECU treatment, and then decreased gradually compared to the untreated cells. However, when cells were treated with EECU and NAC, a ROS scavenger, intracellular ROS production was reversed compared to the EECU-treated cells. This phenomenon was also confirmed by fluorescence microscopy analysis (Figure 5B), indicating that ROS production was accompanied by the induction of apoptosis by EECU.

3.7. *EECU induces the inactivation of PI3K/Akt pathway in T24 cells*

We then examined the effects of EECU on the PI3K/Akt signaling pathway in order to investigate whether EECU-induced apoptosis was affected by PI3K/Akt inactivation, which is indicated by decreased phosphorylation of PI3K and its downstream target molecule Akt, using phosphorylation-specific antibodies. The immunoblotting results demonstrated that EECU remarkably inhibited the phosphorylation of PI3K as well as Akt with increasing EECU concentration (Figure 6A), indicating that they were converted to the inactivated state. Moreover, the addition of LY294002, a specific PI3K inhibitor, enhanced the apoptosis and cytotoxicity induced by EECU (Figure 6B), suggesting that the inactivation of the PI3K/Akt pathway may be involved in EECU-induced cytotoxicity in T24 cells.

3.8. *EECU-induced PI3K/Akt inactivation and growth reduction is ROS-dependent in T24 cells*

To further test whether the contribution of the ROS generation is mediated in the EECU-induced inactivation of the PI3K/Akt pathway, the effects of EECU on the phosphorylation of PI3K and Akt were tested under conditions of artificially blocked production of ROS with NAC. As shown in Figure 7A, the phosphorylation of PI3K and Akt by EECU was greatly restored in the presence of NAC, which means that EECU-induced inactivation of PI3K/Akt pathway is ROS dependent. Furthermore, after T24 cells were co-incubated with EECU and NAC, the apoptosis phenomenon markedly decreased when compared with the corresponding EECU-treated cells (Figure 7B) and reduction in cell viability was also blocked (Figure 7C). These results

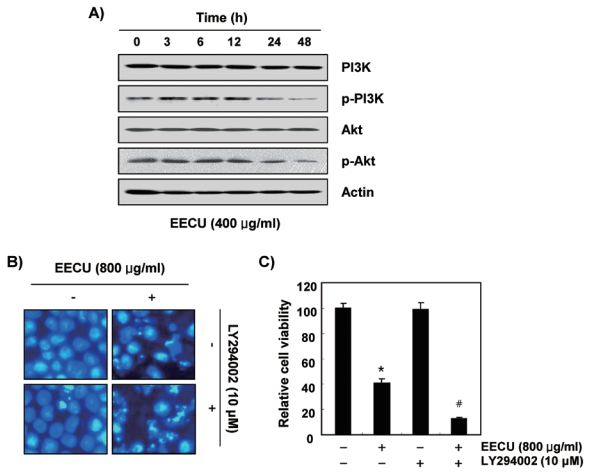

Figure 6. Inactivation of PI3K/Akt pathway by EECU in T24 cells. T24 cells were treated with different concentrations of EECU for 48 h **(A)** or pre-treated with 10 μM LY294002 for 1 h and then treated with 400 μg/mL EECU for a further 48 h **(B** and **C)**. **(A)** Equal amounts of cell lysate were resolved by SDS-polyacrylamide gels, transferred to membranes, and probed with the indicated antibodies. The proteins were visualized using an ECL detection system. Actin was used as an internal control. **(B)** The DAPI-stained nuclei were then observed with a fluorescence microscope (original magnification, ×400). **(C)** The cell viability was measured by an MTT assay. The data are expressed as the mean ± SD of three independent experiments ($^{*}p < 0.05$ *vs*. untreated control; $^{\#}p < 0.05$ *vs*. EECU-treated cells).

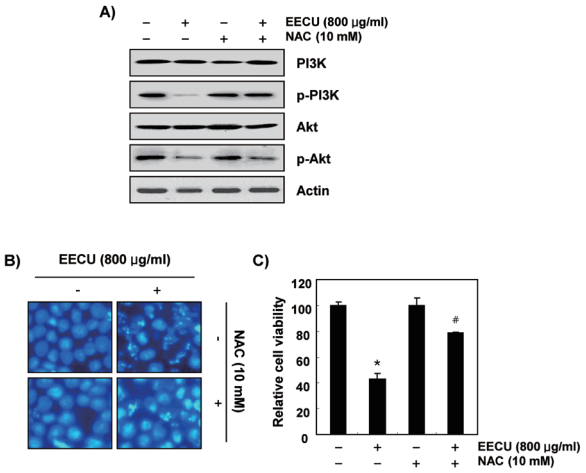

Figure 7. ROS-dependent inactivation of PI3K/Akt pathway in T24 cells. T24 cells were either treated with 800 μg/mL EECU for 48 h or pre-treated with 10 mM NAC for 1 h before EECU treatment and then collected. **(A)** The cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to the membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. Actin was used as an internal control. **(B)** The DAPI-stained nuclei were then observed with a fluorescence microscope (original magnification, ×400). **(C)** The cell viability was determined by an MTT assay. Each point represents the mean ± SD of three independent experiments ($^{*}p < 0.05$ *vs*. untreated control; $^{\#}p < 0.05$ *vs*. EECU-treated cells).

indicate that in addition to the inactivation of PI3K/Akt by EECU, the induction of apoptosis is also ROS dependent.

4. Discussion

In recent decades, considerable interest has been given to discovering anti-cancer drugs from natural products that have been used for a long time for the prevention and treatment of various diseases. Recently, while the possibility of anti-cancer activity of *C. unshiu* peel extract based on the increase of immune activity and anti-inflammatory effect has been raised (*31,32*), little research has been performed on the mechanism of cancer cell proliferation inhibition. In this study, it was demonstrated that EECU treatment induces the apoptosis of human bladder cancer T24 cells. EECU also considerably causes irreversible damage to the cells because the ability to form colonies by the EECU-treated cells was lost. In addition, the anti-cancer effect of EECU depends on the enhanced ROS generation, and the inactivation of the ROS-dependent PI3K/Akt pathway contributes to the effect of EECU on the apoptosis of T24 cells.

Apoptosis is a highly organized and complex physiological process in which cells destroy themselves, which is carried out mainly through two key pathways in response to various extrinsic and intrinsic signals (*7,37*). Caspase-8 and -9 are the major initiators of the extrinsic and intrinsic apoptosis pathway, which results from binding of the cell-surface DRs of the death ligands and mitochondrial perturbation, respectively (*9,11*). Activation of caspase-8 and -9 activates effector caspases such as caspase-3 and -7 through the activation of the caspase cascade system to cause apoptosis by causing degradation of the substrate proteins including PARP, which is a DNA repair enzyme (*38,39*). Thus, the increased activation of caspase-8 and -9 observed in this study indicates that extrinsic and intrinsic pathways may be involved in the induction of apoptosis in T24 cells by EECU. In particular, the increased expression of DR-related proteins by EECU treatment supports the possibility that the extrinsic pathway may be concerned with increased caspase-8 activity. In addition, the loss of mitochondrial membrane integrity was observed with the most typical intrinsic pathway, and the increased translocation of cytochrome *c* from mitochondria to the cytoplasm and increased expression of Bax on Bcl-2 were also observed in the EECU-treated T24 cells. The release of cytochrome *c* requires insertion of mitochondrial membrane and oligomerization of Bax, which is a pro-apoptotic protein belonging to the Bcl-2 family. Thus, the increase in Bax protein expression plays an important role in the activation of the intrinsic pathway, and Bcl-2 is a typical anti-apoptotic protein that suppresses this process (*10,11*).

EECU also down-regulated the IAP family proteins, which selectively bind to caspases and block apoptosis due to their ability to act directly as inhibitors (*40,41*). Increased caspase-3 activity and PARP cleavage were

also observed. Furthermore, the expression of truncated Bid, a pro-apoptotic BH3-interacting domain death agonist, in T24 cells exposed to EECU was increased in an EECU-treated concentration-dependent manner. The truncation of Bid is caused by activated caspase-8, which again facilitates caspase-9 activation (*42,43*). This means that Bid acts as a linker molecule linking DR and mitochondrial dependent pathway. Therefore, the results of this study indicate that the intrinsic pathway activation and the extrinsic pathway were simultaneously involved in the induction of apoptosis of T24 cells by EECU, and that the extrinsic pathway eventually amplified the intrinsic pathway through caspase-8-mediated truncation of Bid.

It is well known that the cellular redox state plays a critical role in regulating cell fate including cell proliferation and death (*12,22*). ROS, typical products of oxidative stress, are mainly produced in mitochondria and function as mediators of various intracellular cascade signaling (*13,14*). In addition, the depletion of mitochondrial permeability transition in the apoptosis induction process of cancer cells by various substances having anti-cancer activity is directly related to the abnormal over-production of ROS (*15*). The attenuation of MMP can induce caspase-9 activation, followed by activation of effector caspases, ultimately resulting in cell apoptosis (*13,14*). One of the major signal transduction systems that closely regulate cell proliferation related to the production of ROS in the cell is a PI3K/Akt signaling pathway, which is abnormally activated or frequently deregulated in a variety of tumors, including bladder cancer, to increase resistance to apoptosis (*17,20,21*). According to our results, the production of ROS in the EECU-treated T24 cells was rapidly increased at an early stage, but was almost completely reversed by NAC, a typical antioxidant. In the T24 cells exposed to EECU, the phosphorylation PI3K and Akt also decreased as the EECU treatment time increased, whereas the total PI3K and Akt protein levels remained constant during all treatments. In addition, apoptosis induced by EECU was further increased when the activity of PI3K/Akt was artificially blocked by a specific pharmacological inhibitor of PI3K/Akt, LY294002, indicating that the inhibition of PI3K/Akt signaling was accompanied by apoptosis induced by EECU. Furthermore, T24 cells cultured in medium containing NAC significantly attenuated EUCC-induced PI3K, Akt dephosphorylation, and cytotoxicity, suggesting that the production of ROS by EECU was a key step in inhibiting the PI3K/Akt pathway in T24 cells.

The above results demonstrate that EECU induces apoptosis in T24 cells through activation of both intrinsic and extrinsic pathways. Meanwhile, EECU-induced apoptosis in T24 cells is mediated by the inactivation of the ROS-dependent PI3K/Akt signaling axis, indicating that the generation of ROS is a potential upstream molecule for PI3K/Akt inactivation and its cytotoxic effect. Our findings provide a new perspective on the molecular mechanism of the inhibitory effect of *C. unshiu* Peel extracts on the growth of cancer cells. Nonetheless, further experiments such as determining the EECU efficacy in other cancer cells, the detection of bioactive compounds of EECU, and animal testing should also be performed.

Acknowledgements

This research was supported by the International Science and Business Belt Program through the Ministry of Science, ICT and Future Planning (2016K000297) and Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korea government (2015R1A2A2A01004633).

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(Received September 5, 2017; Revised October 12, 2017; Accepted October 19, 2017)