**Induction of Apoptosis by *Citrus unshiu* Peel in Human Breast Cancer MCF-7 Cells: Involvement of ROS-Dependent Activation of AMPK**

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The fruit of *Citrus unshiu* Markovich used for various purposes in traditional medicine has various pharmacological properties including antioxidant, anti-inflammatory, and antibacterial effects. Recently, the possibility of anti-cancer activity of the extracts or components of this fruit has been reported; however, the exact mechanism has not yet been fully understood. In this study, we evaluated the anti-proliferative effect of water extract of *C. unshiu* peel (WECU) on human breast cancer MCF-7 cells and investigated the underlying mechanism. Our results showed that reduction of MCF-7 cell survival by WECU was associated with the induction of apoptosis. WECU-induced apoptotic cell death was related to the activation of caspase-8 and -9, representative initiate caspases of extrinsic and intrinsic apoptosis pathways, respectively, and increase in the Bax : Bcl-2 ratio accompanied by cleavage of poly(ADP-ribose) polymerase (PARP). WECU also increased the mitochondrial dysfunction and cytosolic release of cytochrome c. In addition, AMP-activated protein kinase (AMPK) and its downstream target molecule, acetyl-CoA carboxylase, were activated in a concentration-dependent manner in WECU-treated cells. In contrast, compound C, an AMPK inhibitor, significantly inhibited WECU-induced apoptosis, while inhibiting increased expression of Bax and decreased expression of Bel-2 by WECU and inhibition of WECU-induced PARP degradation. Furthermore, WECU provoked the production of reactive oxygen species (ROS); however, the activation of AMKP and apoptosis by WECU were prevented, when the ROS production was blocked by antioxidant N-acetyl cysteine. Therefore, our data indicate that WECU suppresses MCF-7 cell proliferation by activating the intrinsic and extrinsic apoptosis pathways through ROS-dependent AMPK pathway activation.

**Key words** *Citrus unshiu* peel; breast cancer cell; apoptosis; AMP-activated protein kinase; reactive oxygen species

Among various types of programmed cell death associated with cancer cell proliferation inhibition, apoptosis is the most typical cell death mechanism. 1,2 Therefore, inhibition of proliferation of cancer cells through the induction of apoptosis pathway is the most fundamental research area for the discovery of novel anti-cancer agents. Apoptosis accompanied by a specific morphological transformation including the formation of an apoptotic body by chromatin condensation is largely divided into death receptor (DR)-initiated extrinsic and mitochondria-mediated intrinsic pathways. 3,4 The former is induced by binding killing kinetics to the DR and activating the caspase cascade with downstream effector caspases in the upstream initiator caspase-8. The former is induced by the binding of death ligands to the DRs, activating the caspase cascade from upstream initiator caspases such as caspase-8 to downstream effector caspase, including caspases-3 and -7 by recruiting adaptor molecules. 5,6 In contrast, the intrinsic pathway is initiated by mitochondrial dysfunction and activation of caspase-9 by altered expression of Bcl-2 family proteins composed of members capable of promoting or inhibiting apoptosis. 7,8

In addition, such induction of apoptosis is complicated and is controlled by the activation and inactivation of various intracellular signal pathways involved in cell metabolism. One of the molecules of greatest interest recently in inducing apoptosis in cancer cells is 5′-AMP-activated protein kinase (AMPK), a serine/threonine kinase, that plays a critical role in the energy homeostasis of cell. 7,8 AMPK is activated under conditions in which the ratio of AMP is increased compared to ATP depending on various environmental changes in the cell. It has an enzymatic activity to increase the cellular energy level when phosphorylation occurs in the α subunit (Thr 172 residue) among the three subunits (α, β, and γ) constituting AMPK. 9,10 Recently, it has been reported that AMPK may play an essential role in the induction of apoptosis of cancer cells, and that excessive production of reactive oxygen species (ROS) stimulates AMPK activation. 11,12 Although studies on the ROS generation and the role of AMPK are not fully under-
stood, these results suggest that the AMPK may be a potential therapeutic target for inducing apoptotic cell death associated with mitochondrial function impairment.

Plants that have been used worldwide for a long time in traditional medicine have been constantly reviewed as resources for the development of new drugs to control various diseases. In particular, herbal medicines contain a large amount of biologically active substances with little side effects and can be used as an alternative treatment strategy for prevention and treatment of various diseases including cancer. Among them, citrus and dried peels have been used as traditional medicines to treat common colds, bronchial discomfort, and indigestion and have been reported to possess pharmacological effects on inflammation, allergies, diabetes, and viral infections. Preparations of WECU were obtained from Dongeui Korean Medical Center (Busan, Republic of Korea) and identified by Dr. Su-Hyun Hong of Dongeui University College of Korean Medicine. A voucher specimen (WECU-17-1) has been deposited in the Department of Biochemistry, Dongeui University College of Korean Medicine.

Preparation of WECU The dried peels of C. unshiu were provided from Dongeui Korean Medical Center (Busan, Republic of Korea) and identified by Dr. Su-Hyun Hong of Dongeui University College of Korean Medicine. For the preparation of WECU, they were pulverized into a fine powder and then the powder was boiled with distilled water for 3 h. In order to remove insoluble materials, the extract was filtered through a 0.45-mm filter to remove insoluble materials, and the filtrate (WECU) was lyophilized using a vacuum rotary evaporator (BUCHI, Switzerland), and the residue was freeze-dried in a freezing-dryer, and then stored at −80°C. The extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/mL as a stock solution and stored at 4°C. The stock solution was diluted to the desired concentration in the medium before use.

Chromatographic Analysis of WECU To confirmation the reproducibility of WECU, the phytochemical profile of WECU was analyzed using composition analysis based on HPLC using an Agilent 1100 series HPLC instrument (Agilent Technologies, San Jose, CA, U.S.A.) as previously reported. OptimaPak C18 column (RS Tech Co., Daejeon, Republic of Korea) was used for chromatographic separation and gradient elution was carried out using ion exchange water and acetonitrile. HPLC were obtained using UV absorption at 190–400 nm. Standard samples mixtures including naringin, hesperidin, and neohesperidin were dissolved and diluted in methanol at 0.15–200 μg/mL, and the WECU sample was dissolved in methanol at 5 mg/mL. The results of representative sample chromatogram and quantitative analysis obtained from the experiments are shown in Fig. 1.

Cell Culture Human breast cancer MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A.), and were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere with 5% CO2.

Cell Viability Examination To evaluate the cytotoxic ability of WECU, MCF-7 cells were seeded at a density of 2×10⁴ cells per well in 6-well plates. After incubating overnight, the cells were treated with different concentrations of WECU for 72 h. Thereafter, a MTT solution was added to each well at a concentration of 0.5 mg/mL and then incubated at 37°C in a dark environment for 3 h. The MTT solution was removed carefully, and DMSO (200 μL) was added in order to dissolve the formazan complex.

The cell viability was then detected by reading the absorbance of formazan at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Dynatech Laboratories, Chantilly, VA, U.S.A.). The optical density of formazan formed in untreated control cells was used to show 100% viability. The morphological changes were also observed using an inverted light microscope (Carl Zeiss, Oberkochen, Germany).

Detection of Apoptosis via DAPI Staining Detection of apoptosis in MCF-7 cells by WECU, and this phenomenon is ROS production dependent.

MATERIALS AND METHODS

Reagents and Antibodies All tissue culture reagents including RPMI 1640 medium and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5′,6,6′-tetrachloro-1′,3′,3′-tetraethylimidacarbocyanine iodide (JC-I), N-acetyl-l-cysteine (NAC), and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and Bio-Rad protein assay kit were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and Bio-Rad protein assay kit were obtained from (BD Pharmingen (San Diego, CA, U.S.A.) and Bio-Rad Laboratories (Hercules, CA, U.S.A.), respectively). A mitochondrial isolation kit was purchased from Active Motif (Carlsbad, CA, U.S.A.). Colorimetric caspase assay kits and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were obtained from R&D Systems (Minneapolis, MN, U.S.A.) and Molecular Probes (Leiden, the Netherlands), respectively. Primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL) kit were obtained from Amersham Biosciences (Westborough, MA, U.S.A.). All other chemicals not specifically mentioned here were purchased from Sigma-Aldrich Chemical Co.
treatment with WECU, the cells were harvested, washed twice with phosphate buffered saline (PBS), and fixed with 3.7% paraformaldehyde in PBS at 25°C for 10 min. The cells were washed with PBS and stained with DAPI solution (1 mg/mL) for 10 min. The cells were washed with PBS, and morphological changes of the nuclei were examined using a fluorescence microscope (Carl Zeiss).

**Determination of Apoptosis by Flow Cytometry** To calculate the magnitude of the apoptosis by WECU, the cells were harvested, washed with PBS and a binding buffer provided in Annexin V-FITC Apoptosis Detection Kit, and stained with FITC-conjugated annexin V and propidium iodide (PI) for 20 min in the dark. The mixture containing the cells to be analyzed was then analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) according to the manufacturer’s protocol. At least 10000 cells were analyzed from each sample. The annexin $^-$ /PI $^-$ cell population was considered to be cells that did not undergo apoptosis, while the annexin V-FITC $^+$ /PI $^-$ and annexin $^+$ /PI $^+$ cell populations were considered early and late apoptotic cells, respectively.

**Protein Isolation and Western Blot Analysis** After the treatment with WECU, the cells were lysed, and a Bio-Rad protein assay was used to quantify the protein concentration as described in a previous study. At the same time, the mitochondrial and cytosolic fractions were isolated according to the manufacturer’s protocol. For Western blot analysis, the same amounts of protein samples were electrophoretically transferred onto membranes (Schleicher & Schuell, Keene, NH, U.S.A.) following electrophoretic separation on sodium-dodecyl sulfate (SDS) gel. The membranes were blocked with PBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk. They were then probed with specific primary antibodies at 4°C overnight and reacted with the appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. The protein bands were detected using an ECL kit as per the manufacturer’s instructions.

**Measurement of Caspase Activity** The activities of the caspases (caspase-3, -8 and -9) were detected using colorimetric assay kits following the manufacturer’s instructions. In brief, the equal amounts of protein isolated from the cells to be analyzed were incubated with the supplied reaction buffer containing dithiothreitol and Asp-Glu-Val-Asp (DEAD)-p-nitroaniline (pNA), Ile-Glu-Thr-Asp (IETD)-pNA, or Leu-Glu-His-Asp (LEHD)-pNA as substrates for caspase-3, -8 and -9, respectively, at 37°C in the dark. After 2 h of reaction, the reactions were measured by changing the absorbance at 405 nm using an ELISA reader.

**Evaluation of Mitochondrial Membrane Potential (MMP, $\Delta \psi_m$)** Changes in the MMP were assessed using the dual-emission potential-sensitive probe, JC-1. After the treatment with WECU, the cells were collected, washed with cold PBS, and 10 $\mu$M JC-1 dye was loaded at 37°C in the dark. After 30 min, the cells were washed with PBS to remove the unbound JC-1 and the amounts of JC-1 retained by the cells were measured at 488 and 575 nm using a flow cytometer following the manufacturer’s protocol instructions.

**Measurement of ROS Generation** For the detection of intracellular ROS production, DCF-DA dye was used according to the manufacturer’s instructions. Briefly, after collecting the cells treated with WECU for a certain period of time, the cells were collected, rinsed with PBS, and then stained with 10 $\mu$M JC-1 dye was loaded at 37°C in the dark. After 30 min, the cells were washed with PBS to remove the unbound JC-1 and the amounts of JC-1 retained by the cells were measured at 488 and 575 nm using a flow cytometer.

**Data Analysis** The results obtained by performing at least 3 times are expressed as mean±standard deviation (S.D.).
Statistical analyses were performed using the SPSS statistical package (SPSS 13.0 for Windows, SPSS, Inc., Chicago, IL, U.S.A.). Differences between the two groups were analyzed by the two-tailed Student’s t-test, and between three or more groups by one-way ANOVA multiple comparisons. Statistical significance was considered as a difference of \( p < 0.05 \).

**RESULTS**

**Compositional Analysis of WECU**

WECU and three reference compounds (i.e., naringin, hesperidin, and neohesperidin) were subjected to HPLC analysis. The histogram of WECU indicated that three types of flavonoid family chemicals were detected, including naringin, hesperidin, and neohesperidin at 30.32, 31.72, and 33.42 min of retention time, respectively. Quantitative analysis of the above chemicals was conducted, and their quantities were as follows: 0.152 (naringin), 4.460 (hesperidin), and 0.140 (neohesperidin) \( \mu g/mg \) (Fig. 1).

**WECU Inhibited Cell Survival and Induced Apoptotic Cell Death in MCF-7 Cells**

As shown in Fig. 2A, WECU concentration-dependently reduced MCF-7 cell viability, with various cellular morphological changes including membrane blebs, decreased cell density, and increased number of unattached cells (Fig. 2B). To determine whether WECU treatment led to growth inhibition due to apoptosis induction, the effects of WECU on apoptosis induction was studied. The DAPI staining results indicated that nuclear division and chromatin condensation observed in typical apoptosis-induced cells was concentration-dependently increased in WECU-treated cells (Fig. 3A). And, the percentage of annexin V+ /PI− and V+/PI+ cells markedly increased by treatment with WECU compared to that of the untreated control group (Fig. 3B).

**WECU Enhanced Activation of Caspase-8 and -9 and Cleavage of Poly(ADP-ribose) Polymerase (PARP) in MCF-7 Cells**

We next examined whether increased caspase activation is involved in inducing apoptosis in WECU-treated MCF-7 cells. The results of Western blot analysis showed that the expression of pro-caspase-8 and -9 apparently decreased
with increasing WECU concentration, while their active forms increased (Fig. 4A). A subsequent increase in the cleavage of PARP, a representative substrate protein of activated effector caspases was also observed. Consistent with the immunoblotting results, the in vitro activity of caspase-8 and -9 significantly enhanced by the WECU treatment (Fig. 4B).

**Mitochondrial Dysfunction Was Increased in MCF-7 Cells Treated with WECU** Because mitochondrial dysfunction plays a crucial role in intrinsic apoptotic pathway, we next investigated whether the expression of Bcl-2 family proteins was altered by WECU. Among the Bcl-2 family proteins, the key factors controlling the function of mitochondria, Bcl-2 expression remarkably reduced in response to the WECU treatment, but the expression of Bax increased in the WECU-treated cells (Fig. 5A). The reduction of MMP associated with cytoplasmic release of cytochrome C is characteristic of activation of the endogenous apoptotic pathway.

Since the reduction of MMP and release of cytochrome c to cytosol are hallmarks of activation of the intrinsic apoptosis pathway, we further investigated whether this phenomenon is observed in the WECU-induced apoptosis in MCF-7 cells and found that WECU markedly destroys the integrity of the mitochondria measured by concentration-dependent decrease of MMP values (Figs. 5B, C). Subsequently, the cytosolic release of cytochrome c obviously increased upon treatment with increased concentrations of WECU (Fig. 5D).

**AMPK Activation Is Involved in WECU-Induced Apoptosis in MCF-7 Cells** Many previous studies have reported that AMPK activation under stress conditions enhances cancer cell growth inhibition and apoptosis. Thus, we investigated whether WECU activates AMPK, which is reflected by increased phosphorylation of AMPK\(\alpha\) and acetyl-CoA carboxylase (ACC), a downstream target kinase of AMPK. The Western blot results showed that WECU concentration-dependently increased the phosphorylation of AMPK\(\alpha\) (Thr 172) as well as ACC (Ser 79) (Fig. 6A), indicating that AMPK was activated.

Next, the effects of WECU on the levels of Bax and Bcl-2 after the pre-treatment of compound C, an inhibitor of AMPK, were investigated to address whether AMPK activation is involved in the intrinsic pathway for the death of MCF-7 cells induced by WECU. As indicated in Fig. 6B, expression changes of Bcl-2 and Bax decreased or increased by treatment with WECU were prevented in the presence of compound C (Fig. 6B). In line with these observations, the increased population of apoptotic cells was recovered by compound C (Fig. 6C).

**AMPK-Mediated MCF-7 Cell Apoptosis by WECU Was ROS Dependent** Because ROS is a known activator of AMPK, we further examined whether the induction of apoptosis and activation of AMPK is a ROS-dependent signaling pathway. The flow cytometry results using DCF-DA probe demonstrated that the ROS levels increased rapidly within 25 min after the WECU treatment and gradually decreased thereafter compared to the untreated cells. However, the WECU-induced increase in the ROS contents decreased by the addition of NAC, a ROS scavenger (Fig. 7A).

Therefore, we next attempted to investigate whether AMPK...
activation is related to the ROS generation, and ROS production is important for WECU-induced cytotoxic effect. As shown in Fig. 7B, the increase in AMPK phosphorylation by WECU was markedly reduced when artificially blocked ROS production by NAC, indicating that WECU-induced activation of AMPK is ROS dependent. Furthermore, the WECU-induced apoptosis was significantly attenuated by the addition of NAC (Fig. 7C).

**DISCUSSION**

As part of a screening program for identifying anti-cancer active ingredients in traditional medicines, a study was conducted on the mechanism of apoptosis induction by WECU, the ethanol extract of *C. unshiu* peel, in MCF-7 human breast carcinoma cells. The present results suggest that the activation of intrinsic as well as extrinsic apoptosis pathways may be involved in the induction of apoptosis of MCF-7 cells by WECU, and that ROS generation-dependent AMPK activation plays a critical role in this process.

Among the cell apoptosis by two pathways, the extrinsic pathway begins with the activation of caspase-8 due to the recruitment of adaptor molecules upon binding of the cell surface DR of the death ligands.\(^3,4\) In contrast, the intrinsic pathway is initiated by the loss of internal mitochondrial membrane integrity and the activation of caspase-9 by the cytosolic release of pro-apoptotic factors such as cytochrome c from the mitochondria.\(^5,6\) The release of cytochrome c into the cytoplasm requires insertion of mitochondrial membrane and then oligomerization of Bax, a pro-apoptotic protein belonging to the Bcl-2 family. Thus, the increase in the 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 phenomenon.\(^5,6\) Furthermore, the activation of initiator caspases (-8 and -9) in both the pathways in turn induces activation of effector caspases such as caspase-3 and -7, and cleavage of the death substrates.\(^3,4\) The current data clearly showed that the WECU treatment increased Bax expression while Bcl-2 expression was decreased, and subsequently promoted the loss of MMP associated with an increased release of cytochrome c from mitochondria into the cytosol. Additionally, the activation of initiate caspases (-8 and -9) as well as the degradation of PARP, a marker for apoptotic cells and substrate of activated caspase that triggers cellular disassembly and viability reduction,\(^28,29\) was accompanied by the apoptosis induction by WECU. These data suggest that not only the intrinsic pathway but also the extrinsic pathway are involved in the induction of WECU-mediated MCF-7 cell apoptosis. This was also in good agreement with the results of ethanol extract of *C. unshiu* peel in T24 human bladder cancer cells recently published in this laboratory.\(^23\)

Because the AMPK activation is dependent on mitochondrial function, the loss of MMP following the WECU treatment in this study probably implies that mitochondrial func-
tion was impaired in the WECU-treated cells, presumably causing a problem in the intracellular ATP production.8,12) Many studies have shown that the AMPK activation by ATP depletion is directly associated with the apoptosis induced by various stimuli including chemotherapy agents.30–32) AMPK activation requires phosphorylation by upstream AMPK kinases and regulates the activity of various downstream targets including ACC that regulate cell fate.9,10) Therefore, we investigated whether there is a relationship between AMPK pathway and apoptosis of MCF-7 cells by the WECU treatment and found that WECU promotes the phosphorylation of AMPK and its downstream target ACC. However, blockade of AMPK activation by compound C suppressed the WECU-induced increase of Bax expression and the decrease of Bcl-2 expression. In addition, the degradation of PARP by WECU was also partially blocked, and the frequency of apoptotic cells also suppressed to a remarkable level. These data indicate that AMPK activation may be involved in the WECU-induced MCF-7 cell apoptosis, and AMPK is likely to act as an upstream regulator of intrinsic pathway involved in this process.

With the activation of AMPK, the loss of MMP in the apoptosis induction process of cancer cells by various substances having anti-cancer activity is directly connected with the increase in the ROS generation. Many previous studies indicate that ROS acts as a powerful AMPK activator.11,12) Our results indicated that the ROS generation by the WECU treatment increased within 25 min of the WECU treatment and disappeared from MCF-7 cells cultured in medium containing antioxidant NAC. Furthermore, AMPK phosphorylation by WECU markedly suppressed under the conditions of inhibition of ROS production, the degradation of PARP was also attenuated, and the induction of apoptosis also markedly reduced. Although further studies on the inhibition of ATP production and related mechanisms of mitochondrial transport system disturbance are required, these data indicate that the ROS-dependent AMPK activation in MCF-7 cells is a major mediator of apoptosis, and ATP depletion due to mitochondrial damage by WECU probably induces AMPK activity.

In addition to the AMPK pathway, many other intracellular signaling pathways are involved in cancer cell proliferation regulation. One of them is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which has been reported to be overactive in many cancer tissues and has become a target for blocking cancer cell proliferation.33,34) Recently, we have reported that inhibition of ROS-dependent PI3K/Akt signaling is involved in the induction of apoptosis of T24 bladder cancer cells by ethanol extract of C. unshiu peel.23) Therefore, it is anticipated that the production of ROS may play an important role in the induction of cancer cell apoptosis by C. unshiu peel. The C. unshiu peel is composed of various compounds such as polyphenols, terpenoids and flavonoids35) and the anti-cancer potentials of them including the reference compounds (i.e., naringin, hesperidin, and neohesperidin) detected in this study.
are well known. Since these substances are not present only in *C. unshiu* peel, the pro-apoptotic effect of WECU in cancer cells is believed to be due to the overall effect of various substances contained in WECU. Thus, further investigation of the relevance of another intracellular signaling system and the identification of the major active compounds in the WECU should be undertaken in the future.

In summary, our results demonstrate that WECU enhances apoptosis in MCF-7 cells through the activation of the intrinsic as well as extrinsic pathways by enhancing loss of MMP, the cytosolic release of cytochrome c, ROS generation and Bax/Bcl-2 ratio. Furthermore, WECU promoted AMPK activation, and the inhibition of AMPK activity blocked the changes of Bax and Bcl-2 expression by WECU and prevented induction of apoptosis. In addition, when ROS production was blocked, WECU-induced AMPK activation and apoptosis in MCF-7 cells blocked, indicating that ROS is a potential upstream molecule of the cytotoxic effect induced by WECU. Although the search for active ingredients in WECU and their specific anti-cancer activity should be followed, the results indicate that WECU is an interesting natural compound with anti-cancer activity.

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Conflict of Interest The authors declare no conflict of interest.

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