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Relationship between Reactive Oxygen Species and Adenosine Monophosphate-activated Protein Kinase Signaling in Apoptosis Induction of Human Breast Adenocarcinoma MDA-MB-231 Cells by Ethanol Extract of *Citrus unshiu* Peel

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Citrus unshiu peel extracts possess a variety of beneficial effects, and studies on their anticancer activity have been reported. However, the exact mechanisms underlying this activity remain unclear. In the current study, the apoptotic effect of ethanol extract of *C. unshiu* peel (EECU) on human breast adenocarcinoma MDA-MB-231 cells and related mechanisms were investigated. The results showed that the survival rate of MDA-MB-231 cells treated with EECU was significantly inhibited in a concentration-dependent manner, which was associated with the induction of apoptosis. EECU-induced apoptosis was associated with the activation of caspase-8 and caspase-9, which initiate extrinsic and intrinsic apoptosis pathways, respectively, and caspase-3, a representative effect caspase. EECU suppressed the expression of the inhibitor of apoptosis family of proteins, leading to an increased Bax/Bcl-2 ratio and proteolytic degradation of poly (ADP-ribose) polymerase. EECU also enhanced the loss of the mitochondrial membrane potential and cytochrome *c* release from the mitochondria to the cytosol, along with truncation of Bid. In addition, EECU activated AMP-activated protein kinase (AMPK), and compound C, an AMPK inhibitor, significantly weakened EECU-induced apoptosis and cell viability reduction. Furthermore, EECU promoted the generation of reactive oxygen species (ROS), which acted as upstream signals for AMPK activation as pretreatment of cells, with the antioxidant N-acetyl cysteine reversing both EECU-induced AMPK activation and apoptosis. Collectively, these findings suggest that EECU inhibits MDA-MB-231 adenocarcinoma cell proliferation by activating intrinsic and extrinsic apoptotic pathways, which was mediated through ROS/AMPK-dependent pathways.

Key words : AMPK, apoptosis, breast cancer cells, *Citrus unshiu* peel, ROS

Introduction

Breast cancer is known to be one of the most common types of malignancies in women worldwide, and is a leading cause for cancer-related deaths [31]. Although advances in recent therapies have increased the survival rates for women with breast cancer, the incidence and mortality rates for breast cancer in both developed and developing countries have also rapidly increased [18, 24]. It is therefore imperative to understand the basic mechanisms of breast cancer progression, and to find new biological targets and effective treatment strategies for breast cancer prevention and treatment.

Recently, several types of programmed cell death associated with inhibition of the proliferation of cancer cells have been described [28, 32]. Among them, apoptosis, the most typical cell death mechanism, is characterized by the activation of common caspases [5, 23]. The two major effector cascades involved in apoptosis are roughly divided into death receptor (DR)-initiated extrinsic and mitochondria-mediated intrinsic pathways [11, 14]. The extrinsic pathway triggers apoptosis through the binding of death ligand to the DRs, which activates the caspase cascade from the upstream ini-

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tiator caspase-8 to the downstream effector caspases such as caspase-3 and -7 [14, 35]. The intrinsic pathway, also known as the mitochondrial pathway, is regulated by changes in the expression of Bcl-2 family members composed of proteins capable of promoting or inhibiting apoptosis, and the activation of caspase-9 [5, 10]. Such apoptosis induction is regulated by the complicated activation and inactivation of various signal pathways in the cell.

One of the major signaling systems for cell fate control, which plays a key role in the energy homeostasis of cells, is 5′-AMP-activated protein kinase (AMPK) [16, 30]. AMPK is activated under conditions where the AMP:ATP ratio increases with various environmental changes in the cell. When phosphorylation occurs at the Thr 172 residue of α subunit among three subunits, it has enzymatic activity to increase cell energy level [2, 3, 27]. AMPK activation may play a decisive role in the regulation of apoptosis in cancer cells, and excessive reactive oxygen species (ROS) have been reported to stimulate the activation of this kinase [12, 19]. Therefore, the importance of the AMPK signaling pathway has emerged as a potential therapeutic target for inducing apoptosis associated with the inhibition of proliferation of cancer cells.

Over the years, plants that have been used worldwide in traditional medicine have been constantly reviewed as a resource for the development of new drugs for the control of a variety of diseases, including cancer [21, 36]. *Citrus unshiu* Markovich, which belongs to the Rutaceae family, is a seedless and easily peeled citrus fruit that is cultivated in East Asia, including Korea [26, 34]. For thousands of years, citrus and dried peels have been used as traditional medicines to treat common colds, indigestion, and bronchial discomfort [20, 25, 29, 33]. Recently, it was reported that extracts of *C. unshiu* peel reduce tumor growth, which is associated with increased production of cytokines in a tumor-bearing mouse model [17]. In addition, *C. unshiu* peel has been reported to inhibit inflammatory responses in tumor-bearing mice, and reduce the production of pro-cachectic factors in tumors, which was associated with the prevention of skeletal muscle weight loss and atrophy [15]. Nevertheless, the evidence for the anti-cancer effect of *C. unshiu* peel in human cancer cells and the underlying mechanism remain unclear. Therefore, as part of the search for traditional medicinal products with anti-cancer activity, we investigated the anti-cancer activity of ethanol extract of *C. unshiu* peel (EECU) against MDA-MB-231 human breast adenocarcinoma cancer cells. In this study, we found for the first time that AMPK activation by EECU triggers apoptotic cell death in MDA-MB-231 cells, and suggest that ROS production is involved in AMPK activation by EECU.

Materials and Methods

Preparation of EECU

For the preparation of EECU, the dried peels of *C. unshiu* were provided by Dong-eui 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 hr at room temperature. 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℃. The powder was dissolved in dimethylsulfoxide (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 to the desired concentration in the medium before use.

Cell culture

MDA-MB-231 breast adenocarcinoma cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured 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℃ in a humidified atmosphere with 5% CO_2.

Cell viability assay

To assess the effects of EECU on MDA-MB-231 cell viability, the cells were treated with various concentrations of EECU for 24 hr and then the medium was replaced with solutions containing different concentrations of EECU for 24 hr. The cells were treated with 0.1 mg/ml of 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) for 2 hr at 37℃, and the media were carefully removed. The formazan crystals were then dissolved in DMSO. The plate was shaken, and the optical density (OD) of each culture well was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Silicon Valley, CA, USA) at 540 nm. The relative percentage of viable cells was calculated by dividing the ab-

sorbance resulting from the treated cells by that of the control included in each experiment.

Apoptosis analysis

The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Pharmingen San Diego, CA, USA) was used to determine the magnitude of the apoptosis by EECU. Briefly, after collecting cells treated with EECU, the cells were washed with phosphate-buffered saline (PBS) and binding buffer, and then stained with FITC-conjugated annexin V and propidium Iodide (PI) for 20 min in the dark. The mixture was then analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA), according to the manufacturer's protocol. The Annexin V-FITC$^-$/PI$^-$ cell population was considered as normal, while the Annexin V-FITC$^+$/PI$^-$ and Annexin V-FITC$^+$/PI$^+$ cell populations were considered as apoptotic cells.

Western blot analysis

The cells were lysed in a protein extraction buffer (20 mM sucrose, 1 mM ethylenediaminetetraacetic acid, 20 μM Tris-Cl, pH 7.2, 1 mM dithiothreitol, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin), and centrifuged at 15,000 rpm for 30 min at 4℃. In a parallel experiment, the mitochondrial and cytosolic proteins were isolated using a mitochondrial fractionation kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's instructions. Equal amounts of proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel, and were transferred onto polyvinylidene fluoride (PVDF) membrane (Schleicher & Schuell, Keene, NH, USA), using an electrophoretic transfer system. After blocking with TBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk, the membranes were probed with specific primary antibodies at 4℃ 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 by an enhanced chemiluminescence (ECL) kit (Amersham Life Science), following the manufacturer's protocol instructions.

Analysis of caspase activity

The activities of the caspases (caspase-3, -8 and -9) were detected using colorimetric assay kits (R&D Systems, Minneapolis, MN, USA), which utilize 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, according to the manufacturer's instructions. Briefly, the cells were lysed in the supplied lysis buffer. The equal amounts of proteins were incubated with the supplied reaction buffer containing dithiothreitol and DEAD-pNA, IETD-pNA, or LEHD-pNA as substrates at 37℃ for 2 hr in the dark. The degree of enzymatic activity was compared with the changes in absorbance at 405 nm using an ELISA reader.

Analysis of mitochondrial membrane potential (MMP)

The values of MMP were determined with dual-emission potential-sensitive probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1; Sigma-Aldrich Chemical Co). Briefly, the cells treated with EECU were collected, and washed with cold PBS. One hundred ml of 10 μM JC-1 solution was loaded for 30 min at 37℃ in the dark. After, the cells were washed with PBS to remove unbound dye, and the amount of JC-1 retained by 10,000 cells per sample was measured using a flow cytometer (at 488 and 575), by following the manufacturer's protocol.

Analysis of ROS generation

2′,7′-dichlorofluorescin diacetate (DCF-DA, Molecular Probes, Leiden, Netherlands) dye was used to detect intracellular ROS production, according to the manufacturer's instructions. In brief, after collecting the cells treated with EECU for a certain period of time, the cells were harvested, rinsed with PBS, and then stained with 10 μM DCF-DA for 20 min at 37℃ in a dark room. The cells were immediately washed, resuspended in PBS, and analyzed for fluorescence intensity using a flow cytometer. The values were expressed as a percentage of fluorescence intensity relative to blank control cells. To confirm whether intracellular ROS levels play any role in the cytotoxicity of EECU, cells were pretreated with N-acetyl-L-cysteine (NAC, Sigma-Aldrich Chemicals Co.), a well-established antioxidant, for 1 hr prior to treatment with EECU.

Data analysis

The experimental results were presented as mean ± standard deviation (S.D.) of experiments repeated at least three times. In each treatment group, the statistical significance was compared and verified using a one-way ANOVA or

Student *t*-test method ($p<0.05$ or $p<0.01$).

Results

Inhibition of cell viability and induction of apoptosis by EECU in MDA-MB-231 cells

The effect of EECU on MDA-MB-231 cell viability was analyzed by MTT assay. Fig. 1A shows that EECU significantly decreased the MDA-MB-231 cell survival rate in a concentration-dependent manner, and was accompanied by various morphological changes, including membrane blebbing, diminished cell density, and an increased number of floating cells (Fig. 1B). To determine whether EECU treatment led to growth inhibition due to apoptosis induction, the apoptosis rate was measured by a flow cytometer. The results showed that compared with the untreated control group, EECU markedly enhanced the percentage of apoptotic cells (Fig. 1C), indicating that EECU suppressed cell viability by inducing apoptosis in MDA-MB-231 cells.

Activation of caspases and inhibition of expression of IAP family proteins by EECU in MDA-MB-231 cells

Because the activation of caspase cascades plays a key role in two representative apoptosis-inducing pathways [5, 23], we next investigated whether caspase activation is involved in the induction of apoptosis by EECU in MDA-MB-231 cells. Our immunoblotting results showed that expression of pro-caspase-8, an initiator caspase of the extrinsic apoptosis pathway, was apparently decreased with increasing EECU concentration, while expression of active-caspase-8 was increased. Although no expression was observed of the active-caspase-9 or active-caspase-3, an initiator caspase of the extrinsic apoptosis pathway and a typical effector caspase, respectively, the expression of their pro-forms was suppressed, depending on the EECU treatment concentration (Fig. 2A). The expression of inhibitor of apoptosis proteins (IAP) family members, which play a role in inhibiting caspase activity [6, 22], was also decreased by treatment with EECU in a concentration-dependent manner (Fig. 2B). A subsequent increase was also observed in the degradation of poly(ADP-ribose) polymerase (PARP), which is a representative substrate protein of activated caspase-3. Consistent with Western blot analysis results, the *in vitro* activity of the three investigated caspases was significantly enhanced by EECU treatment (Fig. 2C), indicating that both pathways are activated during the induction of apoptosis by EECU.

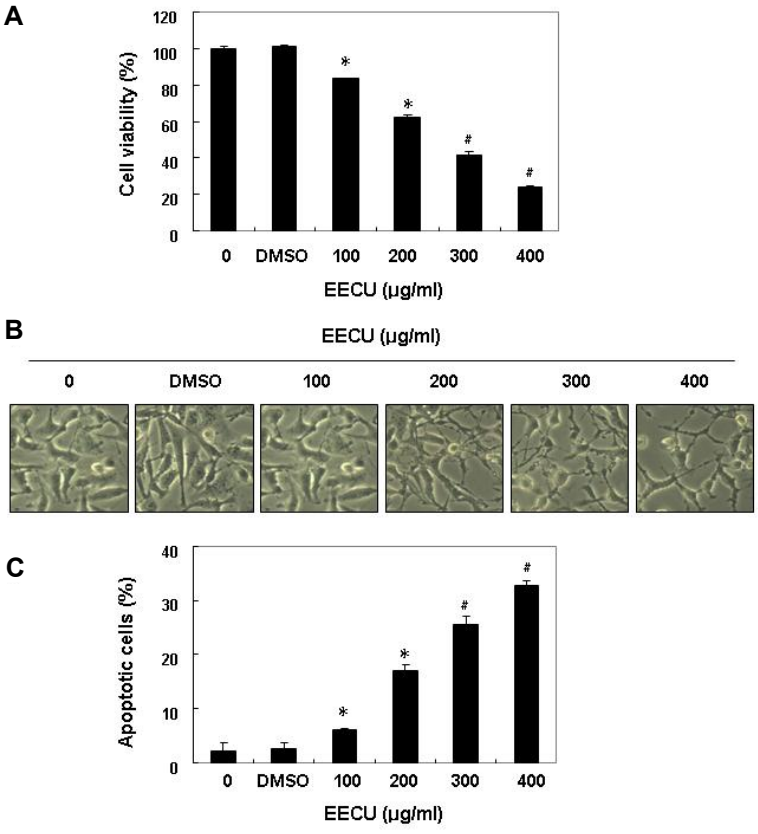

Fig. 1. Inhibition of cell viability and induction of apoptosis by EECU in MDA-MB-231 cells. MDA-MB-231 was treated with various concentrations of EECU for 24 hr. (A) The cell viability was measured by MTT assay. (B) The morphological changes of MDA-MB-231 cells treated with EECU in various concentrations were observed under an inverted microscope (magnification, x200). (C) The percentages of Annexin V-FITC positive cells cultured under the same conditions were indicated. The data were expressed as the mean ± SD of three independent experiments (*$p<0.05$, $^{\#}p<0.01$ vs. untreated control).

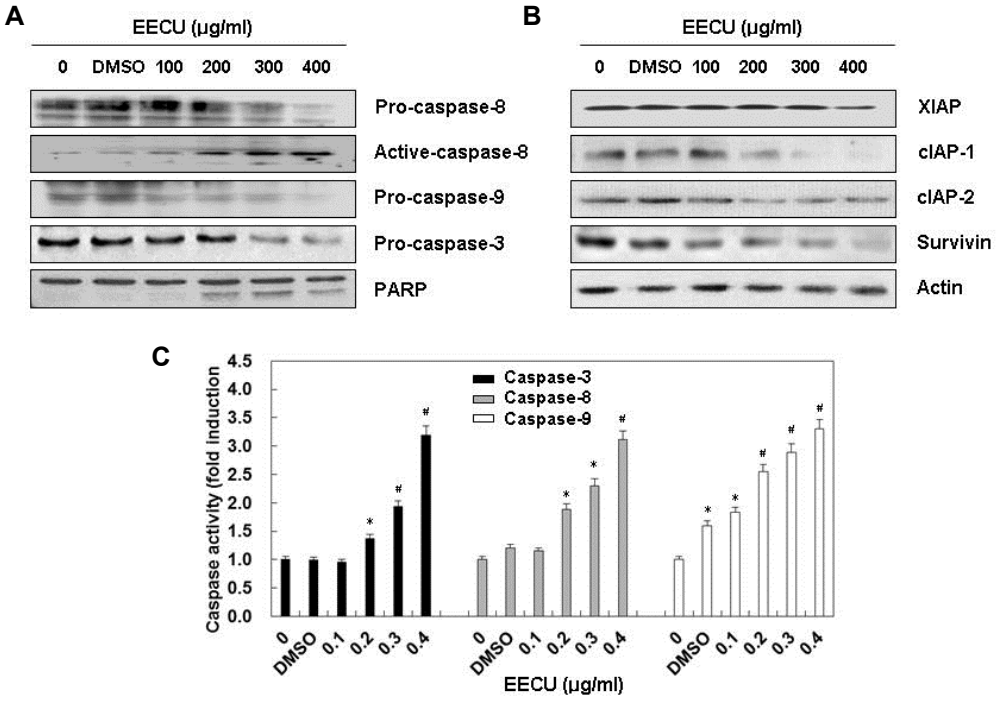

Fig. 2. Activation of caspases and inhibition of IAP family proteins expression by EECU in MDA-MB-231 cells. MDA-MB-231 were treated with the indicated concentrations of EECU for 24 hr. (A and B) The cell lysates were prepared, and equal amounts of cellular proteins were separated on SDS-polyacrylamide 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, $^{\#}p$<0.01 vs. untreated control).

Modulation of DR-related and Bcl-2 family proteins expression by EECU in MDA-MB-231 cells

The results of Fig. 2 indicated that the possibility that two apoptotic pathways were activated in the induction of apoptosis by EECU, so we next investigated the effect of EECU on the expression of DR-related and Bcl-2 family proteins. The immunoblotting data indicated that the expressions of Fas, Fas-associated protein with death domain (FADD), DR4, DR5, and TNF-related apoptosis-inducing ligand (TRAIL) were concentration-dependently increased by EECU treatment, even though the expression of Fas ligand (FasL) was not changed (Fig. 3), suggesting that EECU might regulate the extrinsic pathway. Among the Bcl-2 family proteins, anti-apoptotic Bcl-2 expression was remarkably reduced by EECU treatment, but the expression of pro-apoptotic Bax was increased to some extent. In addition, total Bid expression was decreased by EECU treatment, but truncated Bid (tBid) expression was progressively increased depending on EECU treatment concentration, presumably resulting from truncation by activated caspase-8.

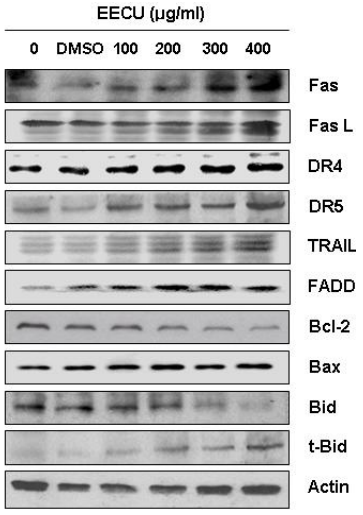

Fig. 3. Effects of EECU on the levels of DR-related and Bcl-2 family proteins in MDA-MB-231 cells. After 24 hr incubation with the indicated concentrations of EECU, the cells were lysed, and cellular proteins were separated by SDS-polyacrylamide gel electrophoresis, and transferred to membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Equal protein loading was confirmed by the analysis of actin in the protein extracts.

Loss of MMP and release of cytochrome c to cytosol by EECU in MDA-MB-231 cells

Recent accumulation studies have shown that the loss of MMP associated with the cytosolic release of cytochrome *c* is a hallmark for the activation of intrinsic apoptosis pathway [10, 35], we therefore investigated whether these phenomena were involved in the EECU-induced apoptosis in MDA-MB-231 cells. Our flow cytometry analysis revealed that EECU markedly destroys the integrity of the mitochondria measured by concentration-dependent loss of MMP (Fig. 4A). Subsequently, the release of cytochrome *c* from the mitochondria into the cytosol was markedly enhanced in a concentration-dependent manner (Fig. 4B, Fig. 4C), indicating that mitochondrial dysfunction may also contribute to EECU-induced apoptosis in MDA-MB-231 cells.

The role of AMPK in EECU-induced apoptosis in MDA-MB-231 cells

Because recent accumulation studies have shown that AMPK activation under stress conditions promotes apoptosis and the growth inhibition of cancer cells [12, 30], we next examined whether EECU activates AMPK, which is reflected by increased phosphorylation of AMPKα and its downstream target kinase acetyl-CoA carboxylase (ACC), using phosphorylation-specific antibodies. Our immunoblotting results demonstrated that EECU remarkably enhanced the phosphorylation of AMPKα (Thr 172), as well as ACC (Ser 79), with increasing EECU concentration (Fig. 5A), indicating that they were converted to the activated state. To address whether AMPK activation is a key pathway for EECU-induced MDA-MB-231 cell apoptosis, the effects of EECU on the expression of caspases and PARP after pretreatment of an inhibitor of AMPK, compound C, were investigated. As shown in Fig. 6B, the EECU-induced down-regulation of caspase-3, -8 and -9, and degradation of PARP were partially prevented in the presence of compound C (Fig. 5B), implying a linkage between caspase and AMPK activation. In addition, the increased apoptotic cell death was attenuated in the presence of compound C (Fig. 5C), and the suppression of cell viability was also significantly abrogated in EECU-treated cells (Fig. 5D). These data indicate that activation of AMPK is important for EECU-induced cytotoxicity in MDA-MB-231 cells.

Induction of apoptosis by EECU through activation of ROS-dependent AMPK in MDA-MB-231 cells

Accumulated evidence has demonstrated that ROS, which are mainly produced by the mitochondria during the execution phase of apoptosis, are a known activator of AMPK [12, 19]. Therefore, we further wanted to determine whether

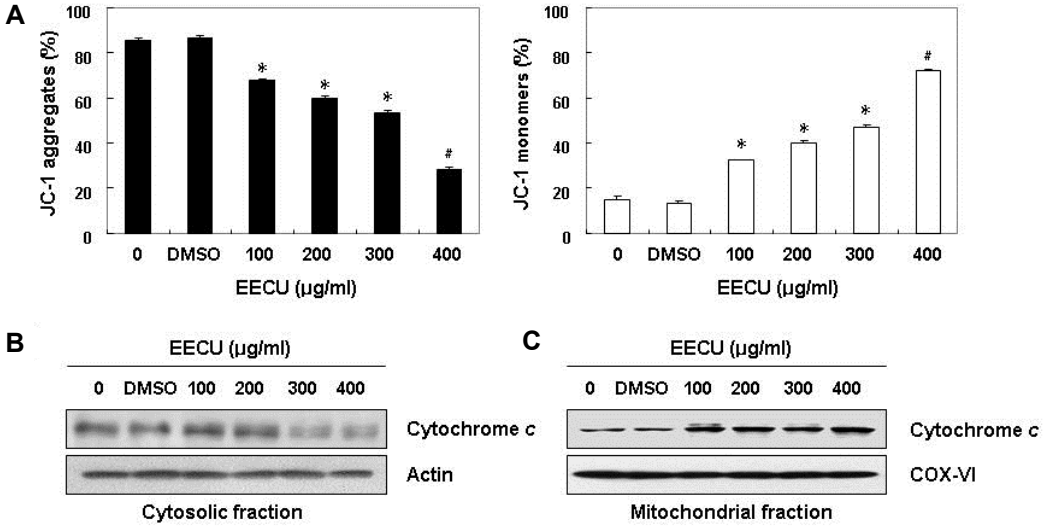

Fig. 4. Effects of EECU on the levels of MMP values and cytochrome *c* expression in MDA-MB-231 cells. (A) After 24 hr incubation with the indicated concentrations of EECU, the cells were stained with JC-1 dye, and were then analyzed on a flow cytometer, in order to evaluate the changes in MMP. The data were expressed as the mean ± SD of three independent experiments (*p<0.05, $^{\#}p$<0.01 vs. untreated control). (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 membranes. The membranes were probed with anti-cytochrome *c* antibody. Proteins were visualized using an ECL detection system. Equal protein loading was confirmed by the analysis of actin and cytochrome oxidase subunit VI (COX VI) in each protein extract.

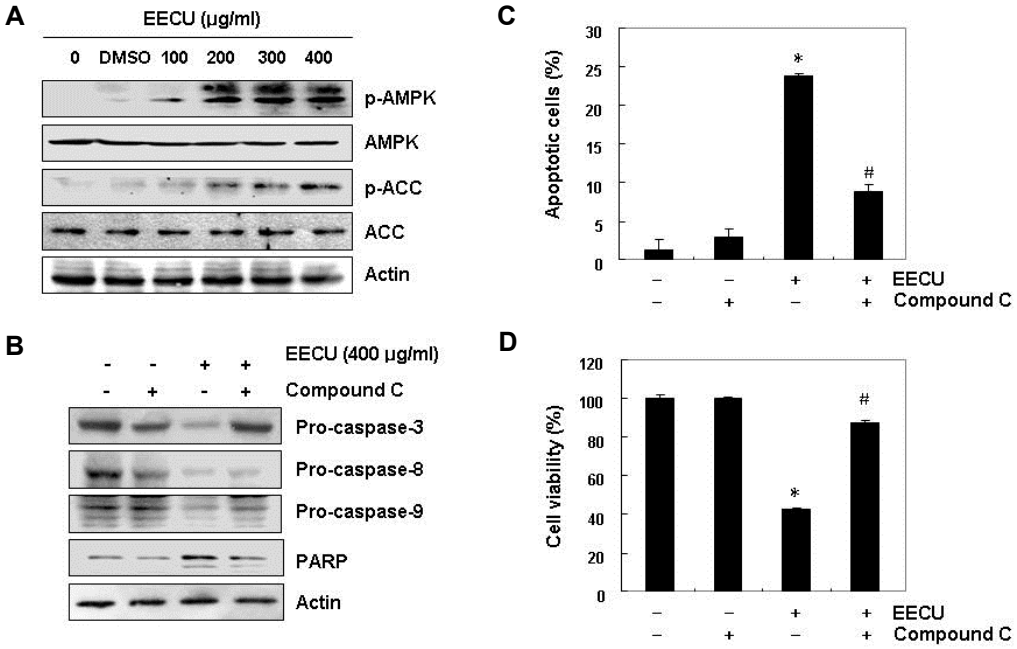

Fig. 5. Involvement of AMPK activation in EECU-induced apoptosis in MDA-MB-231 cells. MDA-MB-231 were (A) treated with different concentrations of EECU for 24 hr, or (B) – (D) pre-treated with 10 μM compound C, an AMPK inhibitor, for 1 hr, and then treated with 400 μg/m EECU for 24 hr. (A and B) Equal 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. (C) The percentages of apoptotic cells were measured using flow cytometric analysis. (D) The cell viability was measured by 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).

the induction of apoptosis by EECU associated with AMPK activation is a ROS-dependent signaling pathway. To determine whether ROS accumulates in cells by EECU, the intracellular ROS levels were measured using DCF-DA probe. As shown in Fig. 6A, the results of flow cytometry indicated that ROS accumulation levels increased within 25 min after EECU treatment, and the levels of ROS generation were progressively reduced relative to untreated cells. However, when the generation of ROS was blocked by NAC, a ROS scavenger, the increase of ROS contents by EECU was greatly reduced.

Next, we attempted to determine whether the activation of AMPK is associated with excessive ROS generation, and ROS generation plays a critical role for the induction of apoptosis by EECU. Fig. 6B demonstrates that EECU-induced phosphorylation of ACC as well as AMPK was greatly attenuated in the presence of NAC. Furthermore, the EECU-induced apoptotic cell death and reduction in cell viability were suppressed when ROS production was artificially blocked (Fig. 6C, Fig. 6C). Taken together, these findings suggest that EECU increases ROS generation, which is required for AMPK activation, and then induces apoptosis in MDA- MB-231 cells.

Discussion

Recently, much attention has focused on the search for anti-cancer active substances that have been used for a long time to treat various diseases. In the present study, we investigated the anti-cancer activity of EECU, ethanol extract of *C. unshiu* peel, and showed that EECU could inhibit the proliferation of MDA-MB-231 adenocarcinoma cells, and induce apoptosis. The principal finding of this study is that both extrinsic and intrinsic apoptosis pathways can be activated to induce apoptosis of MDA-MB-231 cells by EECU (Fig. 7). It also demonstrated that EECU activates AMPK in a ROS-dependent manner, and contributes to EECU-mediated suppression of MDA-MB-231 cell viability.

Of the two typical apoptotic pathways, the DR-mediated extrinsic pathway is activated by binding of the cell-surface DRs of the death ligands to the activation of caspase-8 after the recruitment of the adapter molecules containing FADD, which in turn leads to the activation of caspase-3 and cleavage of death substrates [11, 14]. On the other hand, the in-

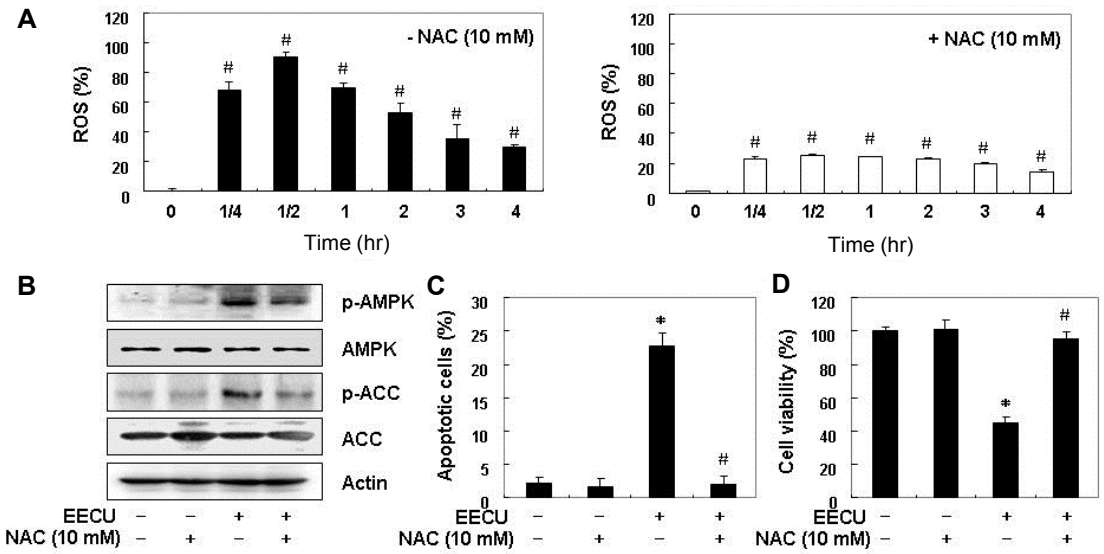

Fig. 6. ROS-dependent activation of AMPK by EECU in MDA-MB-231 cells. (A) MDA-MB-231 cells were either treated with 400 μg/m EECU for the indicated times, or pre-treated with NAC (10 mM) for 1 hr before EECU treatment, and then collected. The medium was discarded, and the cells were incubated at 37℃ 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) The cells were pre-treated with 10 mM NAC for 1 hr, before 400 μg/m EECU treatment. After 24 hr incubation, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis, and transferred to 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 and D) MDA-MB-231 cells were pre-treated with 10 mM NAC for 1 hr, before 400 μg/m EECU treatment for 24 hr. (C) The percentage of apoptotic cells was analyzed by flow cytometer. (D) Cell viability was determined by 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).

ternal pathway is initiated by the activation of caspase-9 with the release of pro-apoptotic factors such as cytochrome *c* from the mitochondria to the cytoplasm following loss of mitochondrial membrane integrity [5, 10]. The insertion of mitochondrial membrane and the oligomerization of Bax, a pro-apoptotic protein belonging to the Bcl-2 family, are required for the release of cytochrome *c*. Therefore, increased expression of Bax plays a key role in the activation of the intrinsic pathway, and Bcl-2 is a typical anti-apoptotic protein that inhibits this process [10, 35]. In addition, the truncation of Bid, a pro-apoptotic BH3-interacting domain death agonist, by activated caspase-8 results in activation of caspase cascade by caspase-9 and caspase-3, following initiation of cytosolic release of cytochrome *c*, which means that Bid acts as a linker molecule that connects the DR and the mitochondria-dependent pathways [1, 13]. In the present study, the activation of caspases (-8, -9 and -3) by EECU clearly demonstrated its apoptotic effects, which was associated with the degradation of PARP, a marker for apoptotic cells and substrate of activated caspase that triggers cellular disassembly and viability reduction [4, 9]. 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 [6, 22]. With the activation of caspases, EECU also increased the expression of FADD, as well as the expression of DR-related proteins, except FasL, suggesting that extrinsic pathway may be involved in apoptosis by EECU in

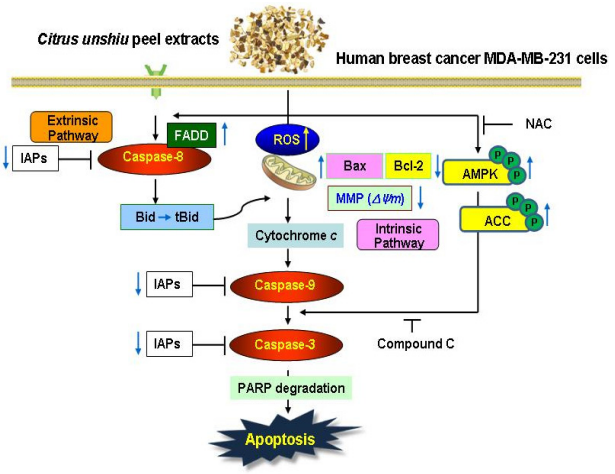

Fig. 7. A suggested schematic model for EECU-induced apoptosis in MDA-MB-231 cells.

MDA-MB-231 cells. Current data also clearly revealed that EECU treatment significantly promotes the ratio of Bax/Bcl-2 expression, followed by a loss of MMP associated with increased release of cytochrome *c* and cleavage of Bid. These results suggest that the intrinsic pathway is also involved in the induction of EECU-induced apoptosis in MDA-MB-231 cells, and that the extrinsic pathway eventually amplifies the intrinsic pathway through caspase-8-mediated truncation of Bid.

Recent studies have shown that the activation of AMPK is directly related to the apoptosis induced by various stimuli, including chemotherapeutic drugs [7, 8]. Activation of AMPK requires phosphorylation by upstream AMPK kinase, and regulates the activity of various downstream targets that regulate cell fate [2, 3]. Therefore, the relation of AMPK pathway to EECU-induced apoptosis in MDA-MB-231 cells was investigated, and it was found that EECU promotes phosphorylation of AMPK and its downstream target ACC. However, blockade of AMPK activation by compound C, an AMPK inhibitor, significantly prevented EECU-induced apoptosis and decreased cell viability, which was accompanied by the reduction of caspase activation and PARP degradation. These data indicate that AMPK activation is involved in EECU-mediated apoptosis in MDA-MB-231 cells, and that AMPK is likely to act upstream of caspase activation in the signaling pathways involved in apoptosis by EECU.

Since mitochondrial electron transport chains are a major source of ATP production, mitochondrial dysfunction promotes the reduction of ATP synthesis, and leads to activation of AMPK, so that the activity of AMPK is dependent on mitochondrial function [12, 30]. In addition, the loss of MMP in the apoptosis induction process of cancer cells by various substances having anti-cancer activity is directly related to the increase of ROS generation, and, as is well known in many previous studies, ROS acts as a powerful AMPK activator [12, 19]. We therefore investigated the possible relevance of ROS production in EECU-induced AMPK activation. As noted in the results, the accumulation of ROS by treatment with EECU disappeared in cultured MDA-MB-231 cells in medium containing anti-oxidant NAC, and markedly attenuated EUCC-induced phosphorylation of AMPK and ACC. Moreover, EECU-induced apoptosis was effectively prevented by blocking ROS production, and the reduced cell viability was also significantly restored to the control level. These results indicate that the activation of ROS-dependent AMPK in MDA-MB-231 cells is a major mediator of apoptosis, and that ATP depletion due to mitochondrial damage caused by EECU probably induced AMPK activity.

The extracts of *C. unshiu* Peel are composed of various phytochemicals such as polyphenols, terpenoids and flavonoids, and their anticancer potentials are well known [15, 17]. However, since these compounds are not present only in *C. unshiu* Peel, the apoptotic effect of WECU in MDA-MB-231 cells seems to be due to the overall effect of various substances contained in WECU. Therefore, further investigation of the relevance of other intracellular signaling pathways and identification of key active compounds in the WECU should be undertaken in the future.

In conclusion, the present results demonstrate that EECU induces apoptosis in MDA-MB-231 adenocarcinoma cells through intrinsic and extrinsic pathways by increasing DR-related regulators, MMP loss, the cytosolic release of cytochrome *c,* and ROS generation, combined with an increase in the Bax/Bcl-2 ratio and Bid truncation. EECU also promoted AMPK activation, and the inhibition of AMPK activity blocked EECU-induced activation of caspase, and prevented the induction of apoptosis. Consequently, when ROS production was blocked, EECU-induced activation of AMPK was inhibited, and the survival rate of MDA-MB-231 adenocarcinoma cells was improved, indicating ROS is a potential upstream molecule for EECU-induced AMPK activation and its cytotoxic effects.

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초록 : 진피 추출물에 의한 인간유방암 MDA-MB-231 세포의 apoptosis 유도에서 ROS 및 AMPK의 역할

김민영[1,2] · 황보현[1,2] · 지선영[1,2] · 홍수현[1,2] · 최성현[3] · 김성옥[4] · 박철[5] · 최영현[1,2]*

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한의학에서는 진피(陳皮)라고 칭하는 귤의 껍질(*Citrus unshiu* peel) 추출물은 항산화, 항염증 및 항균 특성을 포함한 다양한 약리학적 효능을 갖는 것으로 알려져 있다. 최근 그들의 항암 활성에 대한 가능성이 보고되었지만 정확한 기전 연구는 여전히 미비한 실정이다. 본 연구에서는 인간 유방암 MDA-MB-231 세포를 대상으로 진피 에탄올(EECU, ethanol extract of *C. unshiu* peel) 추출물의 항암 효능을 평가하고 그에 따른 기전 연구를 수행하였다. 본 연구의 결과에 의하면 EECU에 의한 MDA-MB-231 세포의 증식억제는 세포사멸(apoptosis) 유도와 관련이 있었다. EECU에 의한 apoptosis는 caspase-8, -9 및 -3의 활성화와 IAPs 계열의 발현 감소에 따른 PARP의 분해와 Bax : Bcl-2 비율의 증가와 연관이 있었다. 또한 EECU는 Bid의 truncation과 함께 미토콘드리아 막 잠재력의 감소와 세포질로 cytochrome *c*의 이동을 촉진시켰다. 아울러 EECU는 AMPK 및 ACC의 인산화를 촉진시켰으나, AMPK 효소 활성의 저해제는 EECU에 의한 apoptosis 유도와 생존력 저하를 현저하게 억제하였다. 부가적으로 EECU는 AMPK 활성화의 상류 신호로 작용하는 활성산소종(ROS)의 생성을 촉진시켰으며, 강력한 항산화제인 NAC는 EECU에 의한 AMPK의 활성화와 apoptosis를 역전시켰다. 결론적으로 EECU는 ROS/AMPK 의존적인 내인성 및 외인성 apoptosis 경로를 활성화시킴으로써 MDA-MB-231 세포 증식을 억제하였음을 알 수 있었다.