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Effect of 2α-Hydroxyursolic Acid on NF-κB Activation Induced by TNF-α in Human Breast Cancer MCF-7 Cells

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Apples are one of the largest contributors of fruit phenolics of all fruits consumed by Americans and contain a variety of bioactive compounds, which have health benefits. Consumption of apples has been linked to reduced risk of chronic diseases such as cancer and cardiovascular disease. Apple extracts have been shown to have the capabilities of inhibiting NF-κB activation in human breast cancer MCF-7 cells. 2α-Hydroxyursolic acid is one of the major triterpenoids isolated from apple peels, and its effects on cell proliferation and TNF-α-induced NF-κB activation in MCF-7 cells were examined. 2α-Hydroxyursolic acid significantly inhibited MCF-7 cell proliferation at doses of 20 μM ($p < 0.05$). Preincubation with 2α-hydroxyursolic acid suppressed TNF-α-induced NF-κB activation in a dose-dependent manner and significantly inhibited the activation at a dose of 20 μM of 2α-hydroxyursolic acid ($p < 0.05$). 2α-Hydroxyursolic acid treatment did not affect the phosphorylation level of NF-κB inhibitor (IκB-α), but proteasome activity in MCF-7 cells was inhibited significantly at doses of 10 and 20 μM ($p < 0.05$). These results suggest that 2α-hydroxyursolic acid has antiproliferative activities against MCF-7 cells and capabilities inhibiting NF-κB activation induced by TNF-α partially by suppressing proteasome activities.

KEYWORDS: Apples; phytochemicals; breast cancer; 2α-hydroxyursolic acid; MCF-7 cells

INTRODUCTION

Major causes of mortality in the United States including cardiovascular disease and cancer are linked to genetic and environmental factors, and diet is the most important one among the environmental factors (*1*−*3*). Significant protective effects of fruits and vegetables against cardiovascular disease and cancer have been found consistently in epidemiological studies (*2, 4*). Fruits are rich in bioactive phenolic compounds such as flavonoids, phenolic acids, stilbenes, coumarins, and tannins (*5*). Consumption of fruits and vegetables that contain high levels of phytochemicals has been reported to have an association with reduced risk of chronic diseases, such as cancer (*5, 6*).

It was expected that about 1,444,920 new cancer cases would be diagnosed and 559,650 Americans might die of cancer in 2007, which accounts for approximately one-fourth of all deaths in the United States (*7*). Breast cancer is a leading cause of female cancer death following lung cancer (*7*), and half of those who develop breast cancer will eventually die of the disease, despite early detection and treatment (*8*). Due to the high death rate of cancer, it is more desirable to prevent the disease before it initiates rather than treat the disease at a later stage.

Nuclear factor κB (NF-κB) is a transcription factor, which regulates expressions of genes related to inflammation, immunity, apoptosis, cell proliferation, and differentiation (*9*). NF-κB exists as homodimers or heterodimers of five subunit proteins including RelA (p65), NF-κB1 (p50/p105), NF-κB2 (p52/p100), c-Rel, and RelB, and p65:p50 heterodimer is the dominant form of NF-κB (*10*). NF-κB is in an inactive form in cytoplasm bound to inhibitory protein, IκB. Once cells are stimulated by extracellular factors, such as pathogens, growth factors, inflammatory cytokines, carcinogens, ultraviolet light, and oxidative stress, NF-κB inhibitor (IκB) is subsequently phosphorylated by IκB kinase (IKK), ubiquitinated by E3 ligase, and degraded by proteasome to release NF-κB (*10, 11*). The released NF-κB moves into the nucleus from the cytoplasm and binds to the promoter region of target genes to activate gene expressions (*10*). Activation of NF-κB target genes results in stimulation in cell proliferation and cell survival pathways, such as increases in cell adhesion, cell migration, and angiogenesis and inhibition of apoptosis. These events promoted tumorigenesis and increased chemoresistance of cancer cells (*11, 12*). Therefore, inhibition of NF-κB activation may reduce the risk of cancer

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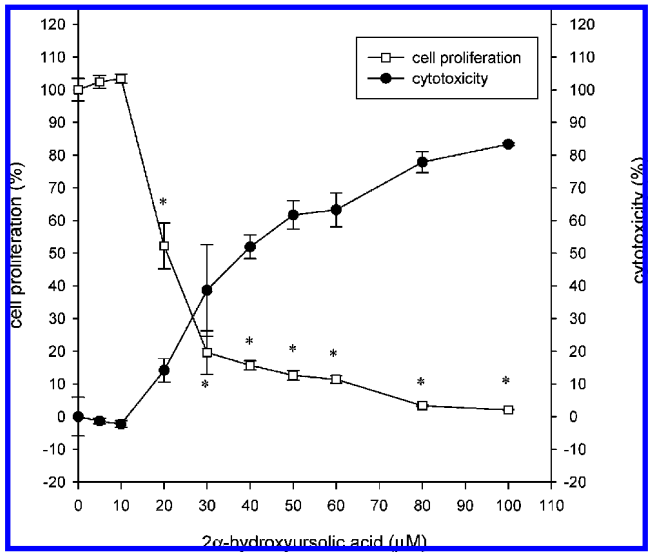

Figure 1. Effect of 2α-hydroxyursolic acid on cell proliferation and cytotoxicity in MCF-7 cells (mean ± SD, $n = 3$). An asterisk indicates significant difference from the control in cell proliferation.

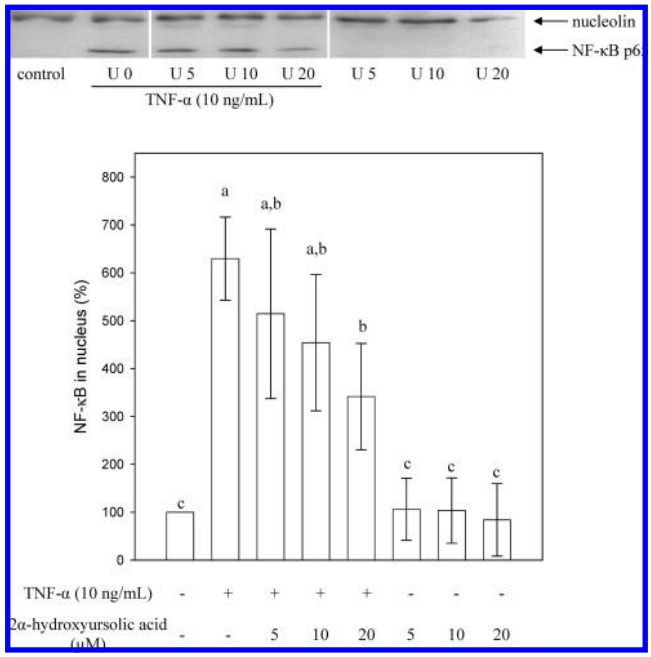

Figure 2. Effect of 2α-hydroxyursolic acid on TNF-α-induced NF-κB activation. MCF-7 cells were treated with 2α-hydroxyursolic acid (0, 5, 10, or 20 μM) at 37 °C for 2 h and, subsequently, incubated in α-MEM with or without TNF-α (10 ng/mL) at 37 °C for 30 min. Nuclear extracts were analyzed with Western blotting assay using antibody to NF-κB p65. Bars with no letters in common are significantly different ($p < 0.05$, mean ± SD, $n = 3$). U, 2α-hydroxyursolic acid (μM).

development and support chemotherapeutics by inhibiting chemoresistance.

Phytochemicals have been suggested to be responsible for the health benefits of fruits and vegetables (*5, 6*). Oxidative stress has been reported to increase the risk of cancer (*13, 14*), and one of the mechanisms of phytochemicals in the prevention of cancer is their antioxidant activities to reduce oxidative stress. Apple is a leading source of fruit phenolics consumed in the United States and is rich in phytochemicals such as phenolics and flavonoid (*15, 16*). Apple consumption was reported to be associated with reduced risk of chronic diseases (*16*) such as cancer (*17*−*19*), cardiovascular disease (*20*), chronic obstructive

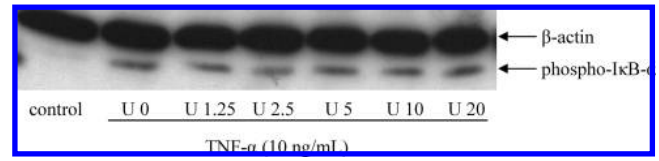

Figure 3. Effect of 2α-hydroxyursolic acid on the phosphorylation of IκB-α. MCF-7 cells were preincubated with 2α-hydroxyursolic acid (0, 1.25, 2.5, 5, 10, or 20 μM) at 37 °C for 2 h and then treated with TNF-α (10 ng/mL) at 37 °C for 30 min. Cytoplasmic fractions were analyzed with Western blotting assay using antibody to phospho-IκB-α. U, 2α-hydroxyursolic acid (μM).

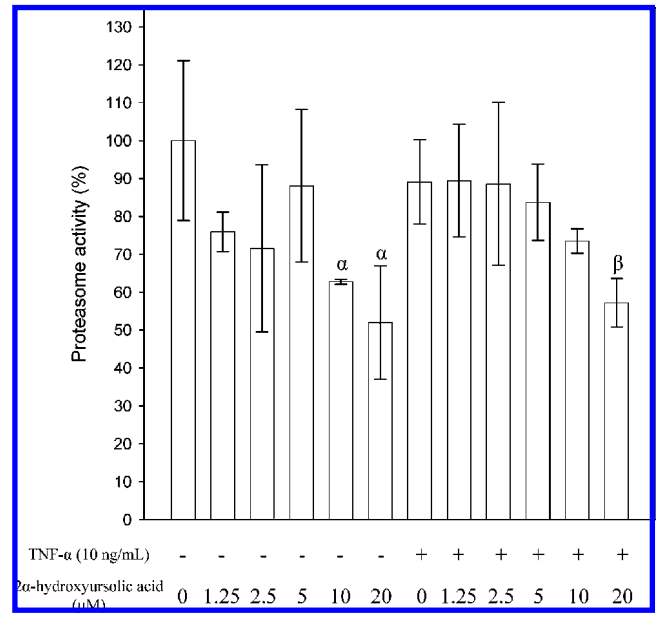

Figure 4. Effect of 2α-hydroxyursolic acid on proteasome activity. MCF-7 cells were pretreated with 2α-hydroxyursolic acid (0, 1.25, 2.5, 5, 10, or 20 μM) at 37 °C for 2 h and then incubated in α-MEM with or without TNF-α (10 ng/mL) at 37 °C for 30 min. Proteasome activities were measured with fluorogenic proteasome substrate. α and β indicate significant difference between control and without or with TNF-α treatment, respectively ($p < 0.05$, mean ± SD, $n = 3$).

pulmonary disease (*21*), and type II diabetes (*22*). Apple phytochemical extracts inhibited the proliferations of colon and liver cancer cells (*15, 23*) as well as breast cancer cells (*24, 25*) in a dose-dependent manner in in vitro studies and prevented mammary cancer growth dose-dependently in a rat model at doses comparable to human dietary consumption of one, three, and six fresh apples a day (*26*). Apple peels have a high antioxidant activity, which is associated with their high content of phenolic compounds and the antiproliferative activity against human liver cancer cells (*27*).

Phytochemicals, such as quercetin (*28*), resveratrol (*29, 30*), curcumin (*25, 31, 32*), epigallocatechin-3-gallate (EGCG) (*33*−*35*), and ursolic acid (*36*), have been reported to inhibit NF-κB activation by blocking steps in the NF-κB signaling pathway of activation. However, the mechanisms of phytochemicals in cancer cell development, which involve NF-κB, are not clear. Our group reported that apple extracts and curcumin significantly blocked TNF-α-induced NF-κB activation in human breast cancer MCF-7 cells by inhibiting proteasomal activity (*25*). However, the specific phytochemicals in apples that are responsible for NF-κB inhibition are not known. Our group has isolated a group of triterpenoids from apple peels and reported that 2α-hydroxyursolic acid, one of the major isolated triterpenoids, had potent antiproliferative activity against

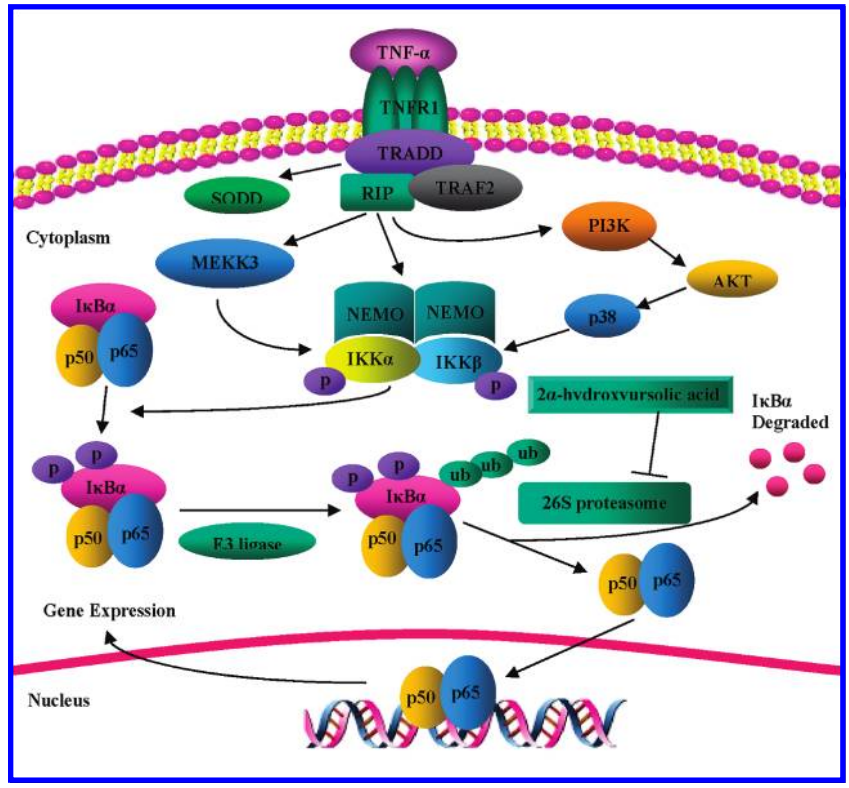

Figure 5. Proposed mechanism of 2α-hydroxyursolic acid inhibiting NF-κB activation induced by TNF-α. Proteasome activity is repressed by 2α-hydroxyursolic acid, and NF-κB is sequestered in cytoplasm bound to IκB-α. TNF-α, tumor necrosis factor α; TNFR1, TNF receptor 1; TRADD, TNFR1-associated death domain; RIP, receptor-interacting protein; TRAF2, TNFR-associated factor 2; MEKK3, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 3; PI3K, phosphatidylinositol 3′-kinase; NEMO, NF-κB essential modulator; IκB-α, inhibitor of NF-κB α; IKK, IκB-α kinase; P, phosphate; ub, ubiquitin.

cancer cell growth (*37*). The objectives of this research were to study the effect of 2α-hydroxyursolic acid, which was isolated from apple peels, on NF-κB activation induced by TNF-α in MCF-7 cells and to investigate the mechanisms of 2α-hydroxyursolic acid to regulate the NF-κB signaling pathway.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in minimum essential medium α medium (α-MEM) (Invitrogen, Carlsbad, CA) containing an additional 10% fetal bovine serum (FBS), 10 mM Hepes, and 10 μg/mL insulin as described previously (*24, 38*).

Chemicals and Antibodies. Apples (Red Delicious variety) were obtained from Cornell Orchards (Ithaca, NY). TNF-α was purchased from Sigma-Aldrich Co. (St. Louis, MO), and proteasome substrate III (Suc-Leu-Leu-Val-Tyr-AMC) and proteasome-specific inhibitor MG-132 were obtained from Calbiochem (San Diego, CA). Methanol, acetone, and ethyl acetate were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ).

Mouse monoclonal antibody to NF-κB was purchased from Abcam (Cambridge, MA), and mouse monoclonal antibody to phospho-IκB-α (Ser32/36) was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich Co., and mouse monoclonal antibody to nucleolin was obtained from Upstate (Charlottesville, VA).

Preparation of 2α-Hydroxyursolic Acid from Apple Peels. Apple phytochemical extracts were prepared using the method reported previously (*39*). 2α-Hydroxyursolic acid was isolated from apple peels and was identified using HR-MS and 1D and 2D NMR as reported previously in our laboratory (*37*).

Treatment of MCF-7 Cells with 2α-Hydroxyursolic Acid and TNF-α. MCF-7 cells were incubated at 37 °C in 5% CO_2 for 24 h following plating in six-well plates (*24, 38*). Cells were rinsed with phosphate-buffered saline (PBS) and were treated with variable doses of 2α-hydroxyursolic acid dissolved in α-MEM. After 2 h of incubation at 37 °C in 5% CO_2, medium was removed and cells were rinsed twice with PBS. Then cells were treated with TNF-α at the concentration of 10 ng/mL dissolved in α-MEM at 37 °C in 5% CO_2 for 30 min, followed by cell harvest. Cells of two wells were used per one treatment.

Western Blotting. For Western blotting analysis, cells were fractionated into nuclear extract and cytoplasmic fraction using the method reported previously (*30, 40, 41*) with modifications by our laboratory (*25*). Cells were rinsed twice with ice-cold PBS and were collected with scraping and centrifugation at 128*g* for 5 min at 4 °C. Cell pellets were suspended in ice-cold hypotonic buffer [10 mM Hepes−KOH (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin] and were kept on ice for 15 min. Nonionic detergent IGEPAL CA-630 (Sigma-Aldrich Co.) was added to the concentration of 1% (v/v), and the cell suspensions were vortexed vigorously for 15 s. Thereafter, the cell suspensions were centrifuged at 12000*g* for 3 min at 4 °C, and the supernatant was collected as the cytoplasmic fraction, which was stored at −80 °C until analysis. The precipitates were suspended in ice-cold high-salt extraction buffer [50 mM Hepes−KOH (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA,1 mM DTT, 10% (v/v) sterile glycerol, 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin] and were rotated at 4 °C for 30 min to facilitate the lysis of nucleus membrane. The lysates were centrifuged at 12000*g* for 30 min at 4 °C, and the supernatants were collected as nuclear extracts, which were stored at −80 °C until analysis as described previously (*25*)

Western blotting analysis was performed as the method described previously (*25, 42*). One hundred micrograms of each sample was subjected to electrophoresis on 10% (w/v) SDS−polyacrylamide gel. After electrophoresis, proteins in gel were transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA) at 4 °C overnight. After

protein transfer, the membrane was blocked with 3% (w/v) nonfat dry milk dissolved in PBS at 4 °C for 2 h. Then the membrane was incubated with primary antibody at 4 °C for overnight. The membrane was subsequently incubated with secondary antibody, which was conjugated with horseradish peroxidase, in PBS containing 3% (w/v) nonfat dry milk at room temperature for 2 h. The blot was revealed using a Phototope HRP Western Blot Detection System (Cell Signaling, Beverly, MA) and sensed by Kodak Biomax MR Film (Kodak, Rochester, NY) after developing and fixing procedures. The bands of proteins were quantified with Labworks gel imaging software (UVP Laboratories, Upland, CA) as described previously (*25*).

Measurement of Cytotoxicity and Inhibition Activity in Cell Proliferation of 2α-Hydroxyursolic Acid. The cytotoxicity of 2α-hydroxyursolic acid on MCF-7 cells was measured with methylene blue assay as reported previously (*43*) with modifications by our laboratory (*25, 44*). MCF-7 cells were plated in 96-well plates at a density of 4×10^4 cells per well and were incubated at 37 °C in 5% CO_2 for 24 h. Cells were, then, treated with various doses of 2α-hydroxyursolic acid dissolved in α-MEM at 37 °C in 5% CO_2 for 24 h. Cells attached on the bottom of wells were counted as viable cells and were stained with methylene blue solution, in which 0.6% methylene blue (BBL, Cockeysville, MD) and 1.25% glutaraldehyde (Sigma-Aldrich Co.) were contained in Hanks Balanced Salt Solution (HBSS) (Invitrogen), at 37 °C for 1 h. Methylene blue stain in cells was eluted with the elution solution, which consisted of 1% (v/v) acetic acid, 49% (v/v) PBS, and 50% (v/v) ethanol, by rotating at room temperature for 1 h. The absorbance at 570 nm was measured by using MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA).

Inhibition activities of 2α-hydroxyursolic acid toward MCF-7 cells proliferation were quantified using the methylene blue assay as described previously (*25*). Briefly, 2.5×10^4 MCF-7 cells were plated in each well of 96-well plate and were incubated at 37 °C in 5% CO_2 for 4 h; then, cells were treated with various doses of 2α-hydroxyursolic acid in α-MEM at 37 °C in 5% CO_2 for 96 h. The remaining cells on the bottom of wells were quantified with methylene blue assay as described above.

Measurement of Proteasome Activity. Proteasome activity was measured using the method previously reported (*25, 45*). The cytoplasmic fraction of cellular proteins was prepared as described above, except that protease inhibitors, such as NaF, Na_3VO_4, PMSF, aprotinin, leupeptin, and pepstatin, were not included in hypotonic buffer. Fifty micrograms of samples was incubated with 100 μM proteasome substrate III in a total of 150 μL of reaction buffer, which is 20 mM Tris-HCl (pH 7.8) containing 0.5 mM EDTA and 0.035% SDS, at 37 °C, and the increase in fluorescence was measured over an interval of 60 min using a Fluoroskan Ascent fluorescence plate reader (Thermo Labsystems, Franklin, MA) with excitation at 355 nm and emission at 460 nm. The experiment was repeated with 1 μM MG-132, and the difference of fluorescence increase between experiments without and with MG-132 was considered to be proteasome activity.

Statistical Analysis. Data were reported as mean ± SD for three replicates, and statistical analysis between treatments was conducted by analysis of variance using Minitab statistical software release 12 (Minitab Inc., State College, PA).

RESULTS

2α-Hydroxyursolic acid at doses of 20 μM and below did not exhibit cytotoxicity toward MCF-7 cells in vitro (**Figure 1**). 2α-Hydroxyursolic acid significantly inhibited MCF-7 cell proliferation at doses of 20 μM and above ($p < 0.05$) (**Figure 1**). The median effect dose (EC_{50}) of 2α-hydroxyursolic acid in the inhibition of MCF-7 cell proliferation was 37.1 ± 0.3 μM, and 20 μM 2α-hydroxyursolic acid inhibited cell proliferation by 47.7%. However, the inhibition of proliferation of 2α-hydroxyursolic acid at 30 μM and above may be due to its cytotoxicity (**Figure 1**).

MCF-7 cells were preincubated with 2α-hydroxyursolic acid at a dose of 5, 10, or 20 μM for 2 h at 37 °C and, subsequently, incubated with TNF-α at a dose of 10 ng/mL at 37 °C for 30 min, which induced NF-κB activation and increased the amount of activated NF-κB p65 in the nucleus over 6-fold. 2α-Hydroxyursolic acid inhibited TNF-α-induced NF-κB activation significantly at a concentration of 20 μM ($p < 0.05$) (**Figure 2**), and 2α-hydroxyursolic acid alone did not change the amount of NF-κB p65 in the nucleus at tested doses (**Figure 2**).

MCF-7 cells were treated with 2α-hydroxyursolic acid at a concentration of 1.25, 2.50, 5.00, 10.00, or 20.00 μM for 2 h at 37 °C, followed by TNF-α treatment for 30 min at 37 °C. Cytoplasmic fractions of cells were analyzed with Western blotting assay using antibody against phospho-IκB-α. 2α-Hydroxyursolic acid did not affect the TNF-α-induced phosphorylation of IκB-α (**Figure 3**).

Effects of 2α-hydroxyursolic acid on proteasome activity of the cytoplasmic fraction were measured after MCF-7 cells were treated with 2α-hydroxyursolic acid at doses of 1.25, 2.50, 5.00, 10.00, or 20.00 μM for 2 h at 37 °C, followed by incubation in α-MEM with or without TNF-α (10 ng/mL) for 30 min at 37 °C using fluorogenic proteasome substrate. 2α-Hydroxyursolic acid at a dose of 10 or 20 μM significantly inhibited proteasome activity ($p < 0.05$) in MCF-7 cells treated without treatment of TNF-α. Twenty micromolar 2α-hydroxyursolic acid significantly reduced proteasome activity in cells treated with TNF-α ($p < 0.05$) (**Figure 4**). TNF-α alone did not affect proteasome activity in MCF-7 cells when compared to control.

DISCUSSION

Apple peels are a waste product of apple sauce and canned apple production (*39*), although they contain a large variety of phytochemicals such as procyanidins, catechin, epicatechin, chlorogenic acid, phloridzin, and quercetin conjugates, which may have potential health benefits (*16, 27, 39*). He and Liu isolated triterpenoids, including 2α-hydroxyursolic acid, from apple peels and showed that most of them had high anticancer activities against HepG2 liver cancer cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells (*37*). In this study, we found that 2α-hydroxyursolic acid, a triterpenoid isolated from apple peels, had activity to inhibit TNF-α-induced NF-κB activation in a dose-dependent manner and repressed proteasome activity, which is an essential step of the NF-κB activation pathway (**Figure 5**). TNF-α induces NF-κB activation with several serial procedures (*46, 47*). Once TNF-α binds to the TNF receptor 1 (TNFR1), the TNFR1 associated death domain (TRADD), receptor-interacting protein (RIP), and TNFR-associated factor 2 (TRAF2) are recruited to TNFR1 phosphorylating and activating IκB-α kinase (IKK). IκB-α, which binds NF-κB, is phosphorylated by activated IKK, and is subsequently ubiquitinated by E3 ligase. 26S proteasome degrades ubiquitinated IκB-α to release NF-κB. The released NF-κB migrates into the nucleus to bind DNA to activate the transcription of inflammatory genes. This study showed that 2α-hydroxyursolic acid did not inhibit phosphorylation of IκB-α in TNF-α-induced NF-κB activation process, which indicated that IKK activation induced by TNF-α was not repressed by 2α-hydroxyursolic acid treatment. Inhibition of proteasome is a potential step, which anticancer therapeutic agents may target, because without proteasome activity breaking down IκB-α, NF-κB is sequestered in cytoplasm bound to the inhibitor, IκB-α, and could not promote the expressions of target genes that are related to inflammation, oxidative stress, and cancer development. PS-341, which is a proteasome inhibitor, has been approved by the FDA for treatment of multiple myeloma (*48*).

NF-κB is involved in a broad range of cell functions, and many studies have been conducted to investigate the functions

of NF-κB in cell growth and death. In cancer cell development, NF-κB modulates the expressions of genes promoting tumor initiation, cell proliferation, antiapoptosis, angiogenesis, metastasis, and chemoresistance, leading to cell resistance and cell survival that facilitate carcinogenesis (*49*). NF-κB activates cyclin D1 expression by binding to multiple cyclin D1 promoter regions, which promotes cell proliferation with G1 to S phase progression (*50*). Bcl-2 family expression is induced by NF-κB (*51, 52*), and p53 stabilization is decreased by NF-κB activation accompanied by induction of Mdm2, a ubiquitin ligase (*53*), which represses apoptotic activities of cells. Chemotherapeutic agents, in many cases, inhibit cancer cells by inducing cell cycle arrest or apoptosis through caspase activation (*54*). In responding to chemotherapeutic agents, cancer cells develop resistance, called chemoresistance, limiting the efficacy of the drugs, and NF-κB activation stimulates cell survival and promotes cancer cell resistance to chemotherapy. Therefore, once chemoresistance develops, higher doses of chemotherapeutic agents are needed to achieve the same efficacy, which brings more cytotoxicity. If the development of chemoresistance is repressed while chemotherapy is performed, the side effects induced by high doses of anticancer drug related toxicity can be minimized.

Our group previously reported that apple extracts and curcumin inhibited NF-κB activation induced by TNF-α in human breast cancer MCF-7 cells and proposed that those phytochemicals can be used as a supporting therapy to lower the chemoresistance (*25*). In this study, we showed that 2α-hydroxyursolic acid, one of phytochemicals isolated from apple peels, has potential activity inhibiting NF-κB activation, and the inhibition of proteasome is partially responsible for the activity. As we indicated previously (*5*), phytochemicals may have additive and synergistic effects responsible for their health benefits. Therefore, 2α-hydroxyursolic acid may partially contribute to the antiproliferative and anti-NF-κB activities of apple peels. Cancer is a complex disease in which multiple steps and many pathways are involved. NF-κB is one of the key pathways affecting cancer development. We have shown that phytochemicals, which can be obtained from ordinary diet, are potent inhibitors of NF-κB activation. By inhibiting NF-κB activation, those phytochemicals may repress cancer cell at stages of initiation and progression and help chemotherapeutic agents to inhibit cancer development reducing chemoresistance. The combinational effects of phytochemicals with chemotherapeutic agents on the inhibition of cancer cell proliferation are under investigation in our laboratory.

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