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Effect of Selected Phytochemicals and Apple Extracts on NF-κB Activation in Human Breast Cancer MCF-7 Cells

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Nuclear factor κB (NF-κB) is a transcription factor, which plays an important role in inflammation, cell proliferation, apoptosis, and immunity in eukaryotes. In cancer cells, NF-κB induces resistance to anticancer chemotherapeutic agents by increasing cell proliferation and inhibiting apoptosis. Therefore, inhibition of NF-κB activation in cancer cells is advantageous in cancer therapy by lowing the resistance to chemotherapy. Several phytochemicals from fruits and vegetables have been reported to inhibit NF-κB activation, but the mechanisms of how the phytochemicals work have not been fully understood. The present study examines the effects of selected phytochemicals and apple extracts on TNF-α-induced NF-κB activation in human breast cancer MCF-7 cells. Apple extracts significantly inhibited the TNF-α-induced NF-κB activation at a dose of 5 mg/mL ($p < 0.05$). Curcumin also significantly blocked the TNF-α-induced NF-κB activation at doses of 10 and 20 μM ($p < 0.05$). Neither apple extracts nor curcumin affected phosphorylation of inhibitor of NF-κB-α (IκB-α); both significantly inhibited proteasomal activity of MCF-7 cells at doses of 2.5 and 5 mg/mL of apple extracts and 20 μM of curcumin ($p < 0.05$). These results suggest that apple extracts and curcumin have the capabilities of inhibiting TNF-α-induced NF-κB activation of MCF-7 cells by inhibiting the proteasomal activities instead of IκB kinase (IKK) activation.

KEYWORDS: Breast cancer; phytochemicals; curcumin; apples; MCF-7 cells

INTRODUCTION

Cancer is the leading cause of death in the United States, second to heart disease (*1*). It was estimated that approximately 1 399 790 new cancer cases would be expected with 564 830 cancer deaths in the United States in 2006 (*2*). Breast cancer is one of the leading causes of female cancer death. About 50% of cancer patients die in spite of the early detection and improvement of treatment. Death due to cancer is mainly caused by metastasis and development of resistance to chemotherapy, and it is difficult to design molecular targets to treat cancers because they change in multiple ways (*3*).

Nuclear factor κB (NF-κB) is a transcription factor that regulates inflammation, immunity, apoptosis, cell proliferation, and differentiation of the cells after binding to DNA and activating gene transcription (*4*). NF-κB is a dimer of five possible subunits, RelA(p65), p50, p52, c-Rel, and RelB, and p65:p50 heterodimer is the predominant form (*5*). NF-κB is bound to an inhibitory protein (IκB-α) in the cytoplasm when it is in an inactive form. Numerous extracellular stimuli including bacteria, viruses, inflammatory cytokines, growth factors, ultraviolet (UV), and oxidative stress cause the phosphorylation of the inhibitory protein IκB-α by IκB kinase (IKK) and subsequently the ubiquitination and degradation of IκB-α by proteasome to release NF-κB. The released NF-κB migrates into the nucleus to bind DNA to activate the transcription of inflammatory and other target genes including COX-2, iNOS, cyclin D1, and Bcl-2 (*6*). In cancer treatment, some of the chemotherapeutic drugs that induce apoptosis lose their activities because they activate NF-κB to induce cancer cell proliferation, which brings chemoresistance to cancer cells (*4*, *7*). NF-κB inhibition can restore the capability of chemotherapeutic agents to repress cancer cells inducing apoptosis. IKK inhibitors prevent phosphorylation of IκB-α, and proteasome inhibitors inhibit degradation of IκB-α, precluding NF-κB activation. Both are essential steps for NF-κB activation.

Epidemiological studies have consistently shown that regular dietary consumption of fruits and vegetables is associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease (*8*−*10*). Whole apple extracts inhibited the growth of colon cancer cells and liver cancer cells in a dose-dependent manner (*11*) and effectively prevented mammary cancer growth in the rat model (*12*). Apple peels have been shown to have a high content of phenolic compounds, antioxodant activity, and antiproliferative activity against cancer cells (*13*). A diet high in fruits and vegetables may prevent oxidative stress and therefore prevent chronic disease and slow aging because they are high in antioxidants and other bioactive

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compounds (*14*, *15*). It was proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for the potent antioxidant activity and anticancer activities and that the complex mixture of phytochemicals in whole foods contributes to the health benefit of fruit- and vegetable-rich diets (*11*, *12*, *15*).

Several phytochemicals including quercetin (*16*), curcumin (*17*), resveratrol (*18*), and tea polyphenol (*19*) were reported to inhibit NF-κB activation (*20*). Antioxidants, such as the phytochemicals mentioned above, inhibit NF-κB activity by scavenging reactive oxygen species, which are used to activate NF-κB, and altering the redox potential to reduce NF-κB DNA binding (*7*). Several phytochemicals were reported to inhibit NF-κB activation by preventing IKK activation (*17*, *18*). However, the mechanisms of phytochemicals to inhibit NF-κB activation are not fully understood. Also, the possible health benefit of apple extracts related to NF-κB is rarely studied, and the mechanism of apple extracts in regulating NF-κB activation is not fully known. The objectives of this study were to investigate the effects of selected pure phytochemicals and apple phytochemical extracts on NF-κB activation induced by TNF-α in human breast cancer MCF-7 cells and to explore the mechanisms of action of apple phytochemical extracts and curcumin to regulate NF-κB activation.

MATERIALS AND METHODS

Cell Culture and Chemicals. MCF-7 cells were maintained in minimum essential medium α medium (α-MEM) (Invitrogen, Carlsbad, CA), which contains 10% fetal bovine serum, 10 mM Hepes, and 10 μg/mL insulin. Apples (Red Delicious variety) were purchased from Cornell Orchard (Ithaca, NY). Quercetin dihydrate, resveratrol, curcumin, epigallcatechin gallate (EGCG), and quercetin 3-β-D-glucoside were obtained from Sigma-Aldrich (St. Louis, MO). Antibody to NF-κB p65 (mouse monoclonal) was purchased from Abcam (Cambridge, MA), and antibody to IκB-α (mouse monoclonal) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to phospho-IκB-α (Ser32/36) (mouse monoclonal) was purchased from Cell Signaling (Danvers, MA).

Preparation of Apple Phytochemical Extracts. Apple phytochemicals were extracted using the method reported previously (*13*, *21*). One hundred grams of flesh and peel of fresh apples without cores was weighed and homogenized with 200 mL of chilled 80% acetone (v/v) using a prechilled Waring blender for 5 min. The sample was further homogenized with a Polytron homogenizer for 3 min. The homogenate was filtered with Whatman no. 2 paper on a Büchner funnel under vacuum, and the filtrate was recovered. The acetone in the filtrate was evaporated using a rotary evaporator at 45 °C until approximately 90% of the filtrate had been evaporated. The filtrate was resuspended in water to the volume of 50 mL. This apple extract has been characterized on the basis of the bioactivity-guided fractionation for antioxidant activity and antiproliferative activity against tumor cell growth, structure identification using HR-MS, and 1D and 2D NMR, and X-ray diffraction analysis we reported previously (*22*). The extracts contained ursolic acid, quercetin-3-*O*-β-D-galactopyranoside, quercetin-3-*O*-α-L-arabinofuranoside, 2α-hydroxyursolic acid, sitosterol-3-*O*-β-D-glucoside, 3β-*trans*-cinnamoyloxy-2α-hydroxyursolic acid, 3β-*trans*-*p*-coumaroyloxy-2α-hydroxyursolic acid, quercetin 3,5,7,3′,4′-pentahydroxyflavonol-3-*O*-β-D-glucopyranoside, and other minor components. The apple extracts contained 191.5 $\pm$ 4.4 mg of phenolics per 100 g of apples, fresh weight. The apple phytochemical extracts were frozen and kept in a −40 °C freezer until use.

Cell Treatment with Phytochemicals and TNF-α. MCF-7 cells were plated in six-well plates at a density of 5×10^5 cells per well and kept at 37 °C in 5% CO_2 for 24 h, and cells in two wells were used together per one treatment. Cells were rinsed with PBS and phytochemical solution in growth medium with no FBS added to the well. After 2 h of incubation at 37 °C in 5% CO_2, medium was removed and cells were rinsed with PBS twice. Following the rinsing, 10 ng/mL of TNF-α solution was added to the well, and cells were incubated at 37 °C in 5% CO_2 for 30 min.

Preparation of Nuclear Extract and Cytoplasmic Fraction of Cells. Nuclear extract and cytoplasmic fraction of cells were prepared using the modified method from the previously reported methods (*18*, *23*, *24*). Cells were rinsed twice with ice-cold PBS and scraped off on the ice. Then, the cell pellet was collected by centrifugation at 128*g* for 5 min at 4 °C. Cells were resuspended in ice-cold hypotonic buffer [10 mM Hepes−KOH (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM Na_3VO_4, 0.4 mM PMSF, 1 mM dithiothreitol, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin] and kept on ice for 15 min. Then, nonionic detergent IGEPAL CA-630 (Sigma-Aldrich Co.) was added to the final concentration of 1% (v/v), and the cell suspension was vortexed vigorously for 15 s. The cell suspension was 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 nuclei pellet was resuspended in the ice-cold high-salt extraction buffer [50 mM Hepes−KOH (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 0.2 mM NaF, 0.2 mM Na_3VO_4, 0.4 mM PMSF, 1 mM dithiothreitol, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin] and was rotated at 4 °C for 30 min to facilitate the lysis of nucleus membrane. Nuclear lysates were centrifuged at 12000*g* for 30 min at 4 °C, and the supernatant was collected as the nuclear extract, which was stored at −80 °C until it was analyzed. The purity of the cytoplasmic fraction and the nuclear extract was evaluated by comparing the relative intensity of the marker proteins, β-actin for the cytoplasmic fraction and HPIα for the nuclear extract, using Western blotting assay. Antibody to β-actin was obtained from Sigma-Aldrich Co., and antibody to HPIα was obtained from Upstate USA, Inc. (Charlottesville, VA).

Measurement of Cytotoxicity. The cytotoxicity of selected phytochemicals and apple extracts was measured by using a methylene blue assay that was reported previously (*25*). MCF-7 cells were plated in a 96-well plate at a density of 4×10^4 cells per well and kept at 37 °C in 5% CO_2 for 24 h. Medium was replaced with growth medium containing selected phytochemicals or apple extracts, and cells were kept at 37 °C in 5% CO_2 for another 24 h. Then, cells were stained with methylene blue solution that contained 1.25% glutaldehyde (Sigma-Aldrich, St. Louis, MO) and 0.6% methylene blue (BBL, Cockeysville, MD) in Hanks Balanced Salt Solution (HBSS) (Invitrogen) at 37 °C for 1 h. Cells were then rinsed with water and dried. Methylene blue stain was eluted with the elution solution that consisted of 49% (v/v) PBS, 50% (v/v) ethanol, and 1% (v/v) acetic acid by agitating the plate at room temperature for 1 h. The absorbance was measured at 570 nm by an MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA).

Measurement of Cell Proliferation. The effects of selected phytochemicals or extracts on MCF-7 cell proliferation were measured using the MTS assay, which was previously described (*26*). MCF-7 cells were plated in a 96-well plate at a density of 2.5×10^4 per well and kept at 37 °C in 5% CO_2 for 4 h. Then, the medium was replaced by the growth medium containing selected phytochemicals or apple extracts, and the cells were incubated at 37 °C in 5% CO_2 for 96 h. At the end of incubation, the medium was removed and cells were treated with MTS solution (Promega, Madison, WI). After 4 h of incubation at 37 °C, the absorbance was measured at 490 nm by an MRX II Dynex plate reader (Dynex Technologies, Inc.).

Western Blotting. The protein concentration of the sample was determined by using a Sigma Total Protein Kit (Sigma-Aldrich), and 100 μg of protein of each sample was subjected to electrophoresis on 10% (w/v) SDS−polyacrylamide gels as reported previously (*27*). After electrophoresis, protein was transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA), and the membrane was blocked with PBS containing 3% (w/v) nonfat milk at 4 °C for 1 h. Then, the membrane was incubated with primary antibody at 4 °C overnight, and it was subsequently incubated with the corresponding secondary antibody, which was conjugated with horseradish peroxidase in PBS containing 3% nonfat milk at room temperature for 1 h. The blot was revealed using a Phototope HRP Western Blot Detection System (Cell Signaling, Beverly, MA) and sensed by Kodak Biomax MR Film

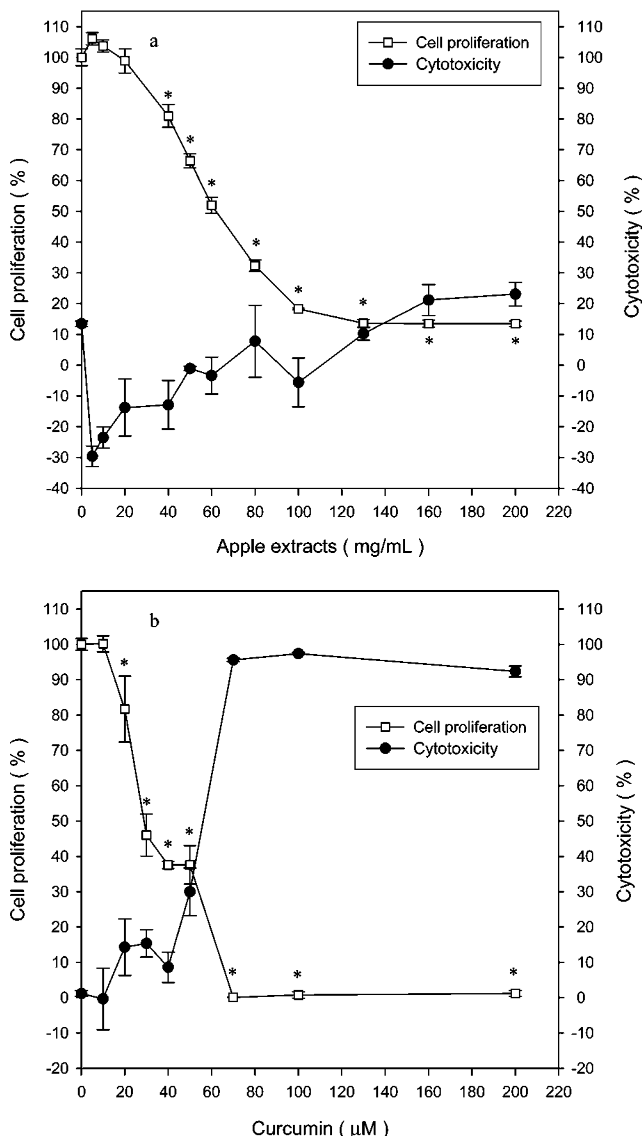


Figure 1. Effects of apple extracts (**a**) and curcumin (**b**) on cytotoxicity and cell proliferation in MCF-7 cells (mean ± SD, $n = 3$). An asterisk (∗) indicates significant difference in cell proliferation compared to the control ($p < 0.05$).

(Kodak, Rochester, NY). The bands of target proteins were quantified using Labworks gel imaging software (UVP Laboratories, Upland, CA).

Proteasome Activity Assay. Proteasome activity in the cytoplasmic fraction was measured by using the method previously reported (*28*). Cytoplasmic fraction was prepared with hypotonic buffer that did not contain protease inhibitors. Fifty micrograms of the cytoplasmic fraction of cells was incubated in 150 μL of reaction buffer [20 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, 0.035% SDS] containing 100 μM Suc-Leu-Leu-Val-Tyr-AMC (Calbiochem, San Diego, CA) at 37 °C. The change in fluorescence over 60 min was measured at 355 nm excitation and 460 nm emission by a Fluoroskan Ascent fluorescence plate reader (Thermo Labsystems, Franklin, MA). The assay was repeated with 1 μM MG-132, a proteasome-specific inhibitor (Calbiochem), to measure protease activities besides proteasome. The proteasome activity was determined by subtracting the fluorescence increase with MG-132 from the one without it.

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

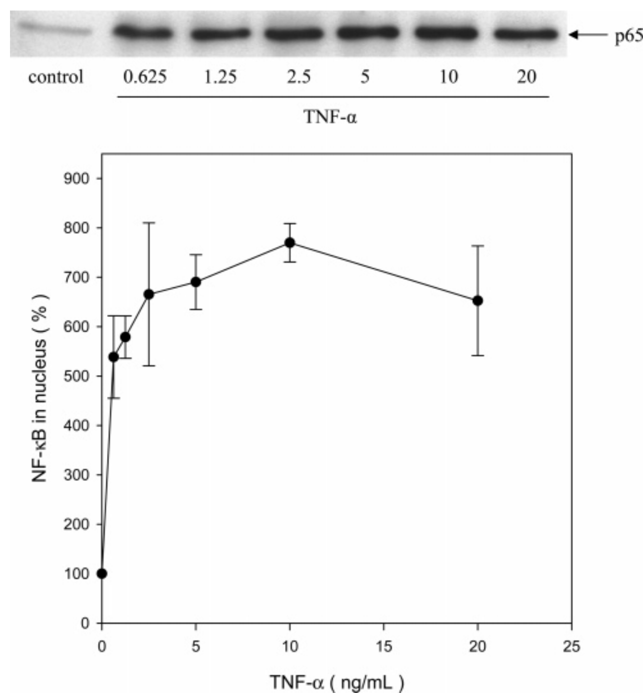


Figure 2. Effect of TNF-α on NF-κB activation. MCF-7 cells were incubated in medium containing TNF-α (0.63, 1.25, 2.5, 10, and 20 ng/mL) for 30 min, and nuclear extracts were analyzed by western blotting using antibody to NF-κB p65 (mean ± SD, $n = 3$).

RESULTS

Apple extracts at doses of 100 mg/mL and below did not exhibit cytotoxicity toward MCF-7 cells in vitro (**Figure 1a**). Apple extracts significantly inhibited MCF-7 cell proliferation at concentrations of 40 mg/mL and above ($p < 0.05$) in a dose-dependent manner. The median effect dose (EC_{50}) of apple extracts in the inhibition of MCF-7 cell proliferation was 65.1 ± 1.5 mg/mL (**Figure 1a**). At the concentration of 100 mg/mL, apple extracts inhibited MCF-7 cell proliferation by 81.7% ($p < 0.05$). Curcumin did not exhibit cytotoxicity toward MCF-7 cells in vitro at a concentration of 40 μM or below, but the cytotoxicity of curcumin was obvious at a concentration of 50 μM or above ($p < 0.05$) (**Figure 1b**). Curcumin significantly inhibited MCF-7 cell proliferation at doses of 20 μM and above ($p < 0.05$) in a dose-dependent manner (**Figure 1b**), and the EC_{50} (the concentration inhibiting the cell proliferation by 50%) was 27.2 ± 1.7 μM. At the concentration of 40 μM, curcumin inhibited MCF-7 cell proliferation by 62.4% ($p < 0.05$).

Effects of doses of TNF-α to activate NF-κB in MCF-7 cells were investigated. MCF-7 cells were incubated in various doses of TNF-α (0.63, 1.25, 2.5, 5, 10, and 20 ng/mL) dissolved in α-MEM with no FBS at 37 °C for 30 min. NF-κB p65 protein in nuclear extract was assessed by western blotting using antibody to NF-κB p65. TNF-α activated NF-κB in MCF-7 cells in a dose-dependent manner (**Figure 2**). NF-κB activation was stimulated by as little as 0.63 ng/mL ($p < 0.05$) and began to plateau at a concentration of ≥2.5 ng/mL. At a concentration of 10 ng/mL, TNF-α increased the amount of NF-κB p65 in the nucleus by >6-fold ($p < 0.05$). Therefore, the dose of 10 ng/mL of TNF-α was selected as a standard treatment to activate NF-κB in MCF-7 cells.

Effects of incubation of MCF-7 cell in selected phytochemicals and apple extracts on TNF-α-induced NF-κB activation were studied (**Figure 3**). MCF-7 cells were incubated in α-MEM with no FBS containing various doses of apple extracts (1.25, 2.50, and 5.00 mg/mL), curcumin (1.25, 2.50, 5.00, 10.00, and

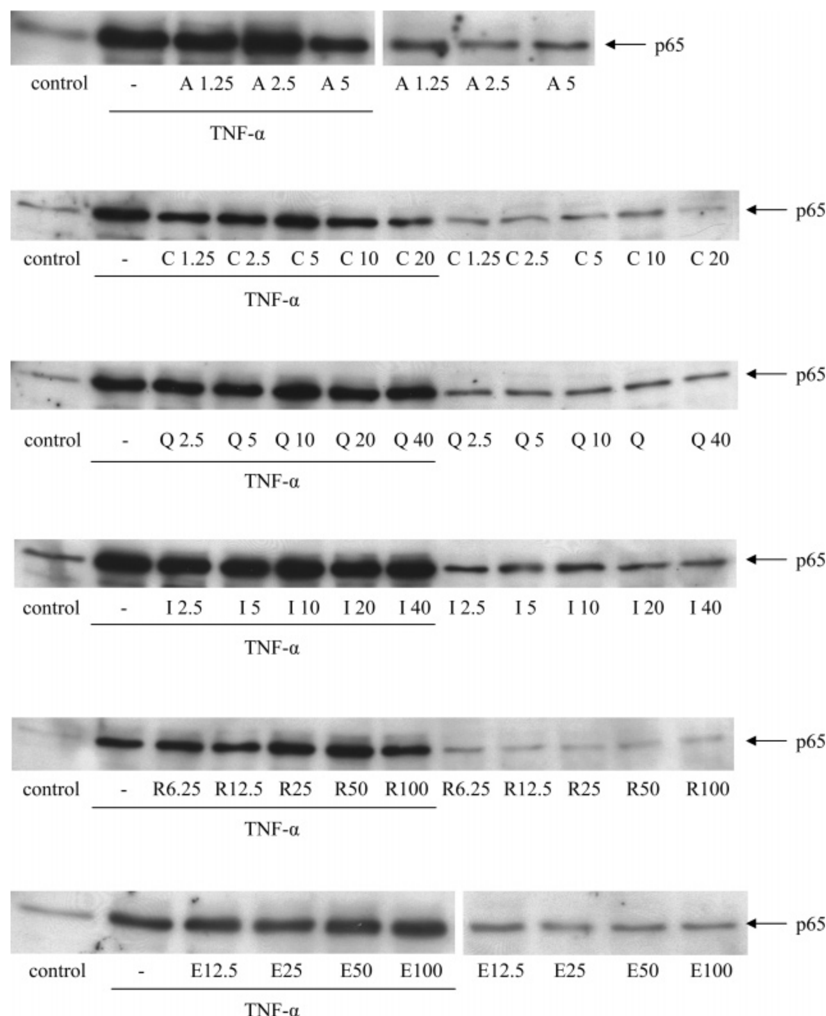


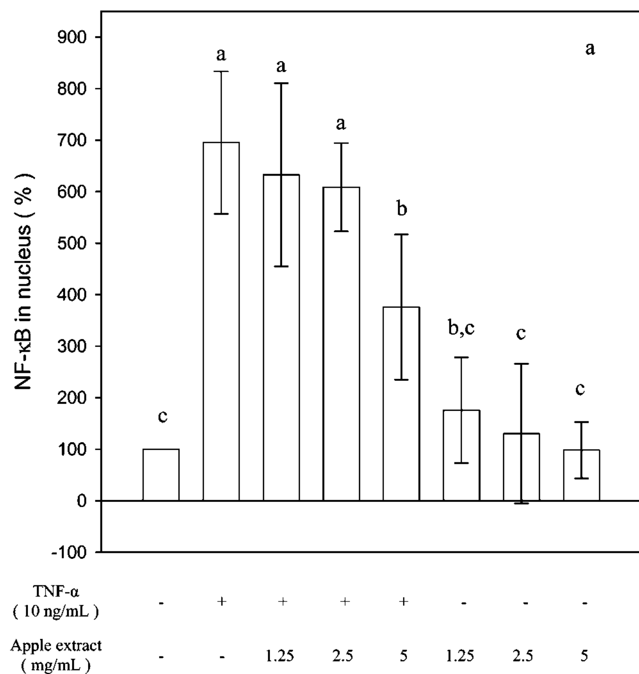
Figure 3. Effects of phytochemicals on TNF-α-induced NF-κB activation. MCF-7 cells were incubated in medium with apple extracts (1.25, 2.5, and 5 mg/mL), curcumin (1.25, 2.5, 5, 10, and 20 μM), quercetin dihydrate (2.5, 5, 10, 20, and 40 μM), quercetin 3-β-D-glucoside (2.5, 5, 10, 20, and 40 μM), resveratrol (6.25, 12.5, 25, 50, and 100μM), or EGCG (12.5, 25, 50, and 100 μM) for 2 h followed by TNF-α treatment (10 ng/mL) for 30 min. Nuclear extracts were assessed by western blotting using antibody to NF-κB p65. A, apple extracts; C, curcumin; Q, quercetin dihydrate; I, quercetin 3-β-D-glucoside; R, resveratrol; E, EGCG.

20.00 μM), quercetin dihydrate (2.5, 5, 10, 20, and 40 μM), quercetin 3-β-D-glucoside (2.5, 5, 10, 20, and 40 μM), resveratrol (6.25, 12.5, 25, 50, and 100 μM), or EGCG (12.5, 25, 50, and 100 μM) at 37 °C for 2 h, followed by 30 min of reaction with 10 ng/mL of TNF-α at 37 °C. The amount of NF-κB p65 in the nucleus was quantified by western blotting and image analysis. At the dose of 5 mg/mL, apple extracts significantly prevented TNF-α-induced NF-κB p65 increase in the nucleus ($p < 0.05$) (**Figure 4a**). Apple extracts did not affect the phosphorylation of IκB-α (**Figure 5**). Curcumin significantly inhibited TNF-α-induced NF-κB p65 increase in the nucleus at concentrations of 10 and 20 μM ($p < 0.05$) (**Figure 4b**). Curcumin did not affect the phosphorylation of IκB-α either (**Figure 5**). Both apple extracts and curcumin prevented the increase of NF-κB p65 induced by TNF-α in a dose-dependent manner. Quercetin dihydrate, quercetin 3-β-D-glucoside, resveratrol, and EGCG did not change TNF-α-induced NF-κB p65 increase in the nucleus at the doses tested.

The proteasome activity of the cytoplasmic fraction of MCF-7 cells was measured after cells were incubated in apple extracts (0.31, 0.63, 1.25, 2.50, and 5.00 mg/mL) or curcumin (1.25, 2.50, 5.00, 10.00, and 20.00 μM) for 2 h followed by the treatments with or without 10 ng/mL of TNF-α for 30 min at 37 °C (**Figure 6**). Incubation in 2.5 and 5 mg/mL of apple extracts or 20 μM curcumin significantly lowered proteasome activity when compared to the control ($p < 0.05$). TNF-α treatment did not change proteasome activity significantly when compared to the control. Incubation in 2.5 and 5 mg/mL of apple extracts or 10 and 20 μM curcumin followed by TNF-α treatment significantly lowered proteasome activity when compared to the control with TNF-α treatment ($p < 0.05$).

DISCUSSION

Cancer is a disease with cell proliferation out of control. Cancer chemotherapeutic agents mainly target cell proliferation and induce cancer cell death. However, in response to the anticancer agents, cancer cells develop resistance by activating NF-κB to increase cell proliferation and to decrease apoptosis. Cells may require high doses of anticancer agents to treat the cancer. High doses of the chemotherapeutic agents may bring side effects to normal cells besides cancer cells. Therefore, it has the advantage to increase the efficacy of anticancer agents and reduce their toxicity by inhibiting NF-κB activation associated with cancer treatment. Previously, our group reported that fruits and vegetables that are consumed on a daily basis inhibited cancer cell proliferation (*11*, *29*, *30*). Recently we reported that whole apple extracts effectively inhibited mammary cancer in



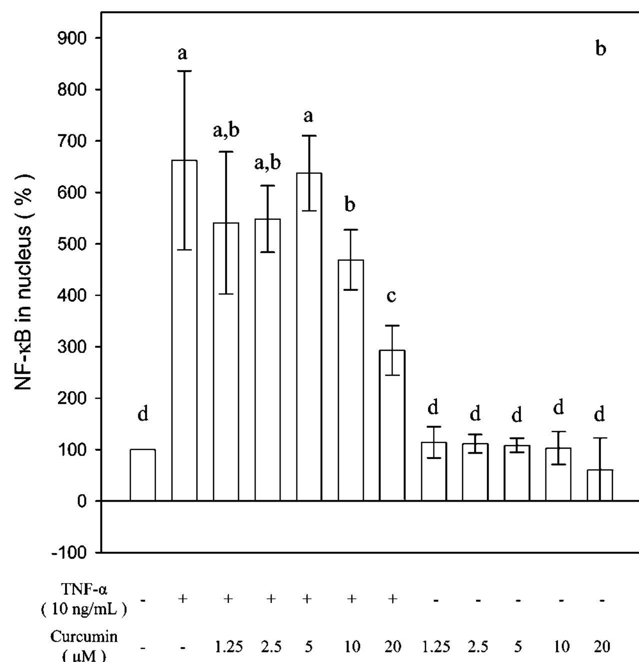


Figure 4. Effects of apple extracts (**a**) and curcumin (**b**) on TNF-α-induced NF-κB activation. Incubation in medium with apple extracts or curcumin for 2 h was followed by treatment with or without 10 ng/mL of TNF-α for 30 min at 37 °C. Nuclear extracts were assessed by western blotting using antibody to NF-κB p65. Bars with no letters in common are significantly different ($p < 0.05$, mean ± SD, $n = 3$).

a rat model in a dose-dependent manner at doses comparable to human consumption of one, three, and six apples a day (*12*). In this study, we demonstrated that apple extracts and curcumin inhibited MCF-7 cell proliferation significantly at concentrations of 40 mg/mL and 20 μM, respectively. Both apple extracts and curcumin at these concentrations inhibited cell proliferation without cytotoxicity. This result was consistent with our previous finding that apple extracts had potent anticancer activity both in vitro (*11*, *13*, *30*) and in vivo (*12*).

It was reported that NF-κB induced the expressions of specific genes to promote cancer cell growth (*31*). NF-κB up-regulates

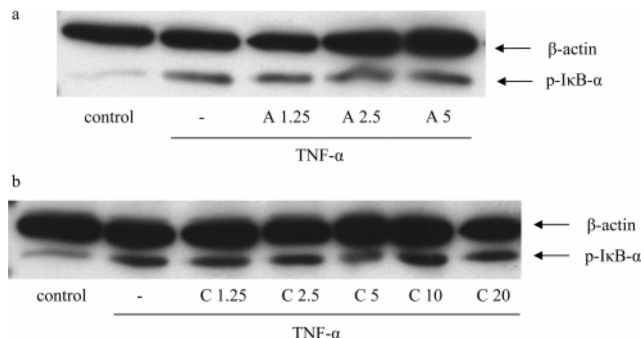
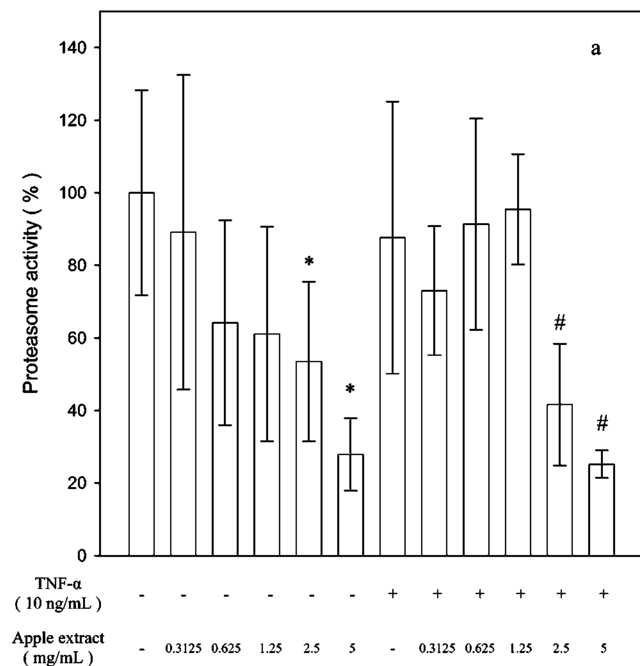


Figure 5. Effects of phytochemicals on the phosphorylation of IκB-α. MCF-7 cells were incubated in medium with apple extracts (**a**) (1.25, 2.5, and 5 mg/mL) or curcumin (**b**) (1.25, 2.5, 5, 10, and 20 μM) for 2 h, followed by TNF-α treatment (10 ng/mL) for 30 min. A, apple extracts; C, curcumin. Cytoplasmic fractions were analyzed by western blotting using antibodies to p-IκB-α and β-actin.

the gene expressions of Bcl-xL and cyclin D1 and activates the c-Myc oncogene promoter, which lead to cancer cell proliferation (*32*−*34*). NF-κB inhibits p53 activity by inducing its inhibitor, Mdm2; therefore, NF-κB can block apoptosis that is induced by p53 (*35*). Inhibition of NF-κB activity may help chemotherapy by restoring apoptosis in the cells. Several phytochemicals including curcumin (*17*), quercetin (*16*), resveratrol (*18*), and EGCG (*19*) were reported to block NF-κB activity. In this study, we established a model for NF-κB activation by TNF-α and tested the effects of selected phytochemicals on NF-κB activation in human breast cancer MCF-7 cells. Only apple extracts and curcumin significantly blocked the NF-κB activation induced by TNF-α in MCF-7 cells, and no other phytochemicals tested alone affected NF-κB activation. The mechanism of TNF-α-induced NF-κB activation is well established (*6*). First, TNF-α recruits TNF-receptor-associated death domain protein (TRADD), receptor-interacting protein (RIP), and TNF-receptor-associated factor 2 (TRAF2) to the TNF receptor 1 (TNFR1) sequentially, which is followed by activation of IKK. Activated IKK, which is phosphorylated, phosphorylates IκB-α on Ser32 and Ser36. Phosphorylated IκB-α is ubquitinated by enzymatic cascade including E1, E2, and E3 and subsequently degraded by 26S proteasome (*36*). Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase 3 (MEKK3) is required for IKK activation (*37*). Phosphatidylinositol 3′-kinase (PI3K) and serine/threonine kinase AKT also play critical roles in IKK activation and NF-κB activation (*38*). Because MEKK3 and AKT pathways are the main mechanisms for IKK activation, if those pathways are blocked, IKK and NF-κB activation will be subsequently inhibited. It was reported that curcumin inhibited TNF-α-induced AKT activation and its association with IKK in U937 (human myeloid leukemia) cells, which leads to inhibition of NF-κB activation (*39*). TNF-α-induced NF-κB activation by the PI3K/AKT pathway is a cell type specific mechanism (*40*). Because AKT phosphorylates and activates IKKα, in the case of a low ratio of IKKα to IKKβ in the cell, inhibition of the PI3K/AKT pathway is not enough to block TNF-α-induced NF-κB activation. We found that apple extracts or curcumin did not block phosphorylation of IκB-α with the doses that blocked NF-κB activation in MCF-7 cells, which indicated that IKK activation was not inhibited by apple extracts or curcumin and that they blocked NF-κB activation through inhibiting steps other than the MEKK3 or PI3K/AKT pathway.

In the present study, apple extracts and curcumin inhibited proteasome activity in a dose-dependent manner, which can be



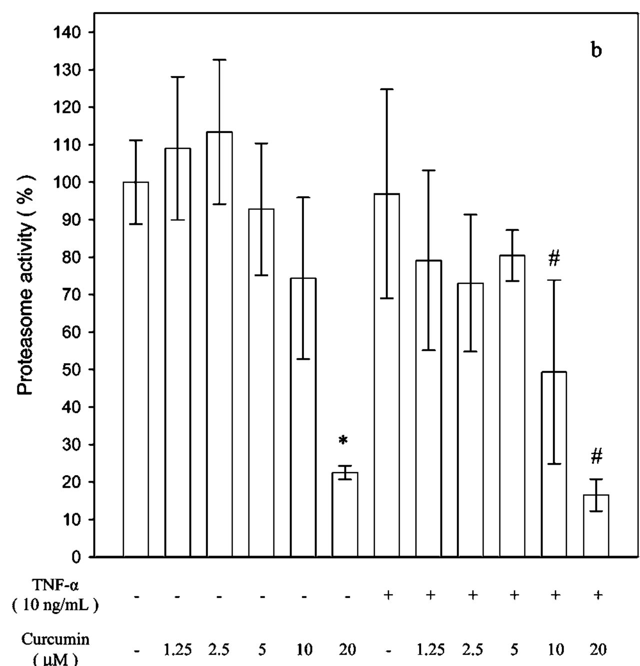


Figure 6. Effects of phytochemicals on proteasome activity. MCF-7 cells were incubated in medium with apple extracts (**a**) or curcumin (**b**) for 2 h, followed by incubation in medium containing no TNF-α or 10 ng/mL of TNF-α for 30 min. Proteasome activities in cytoplasmic fraction were measured using fluorogenic substrate of proteasome. ∗ and # indicate significant difference from the controls without or with TNF-α treatment, respectively ($p < 0.05$, mean ± SD, $n = 3$).

one of the mechanisms to block TNF-α-induced NF-κB activation. Proteasome is one of the targets to regulate NF-κB activation. Inhibitors of 26S proteasome block the degradation of IκB-α and keep NF-κB bound to IκB in the cytoplasm. Therefore, the inhibition of proteasome leads to cell cycle arrest and apoptosis, which are the results of the inhibition of NF-κB activation.

Altogether, apple extracts and curcumin blocked NF-κB activation induced by TNF-α through inhibiting proteasome activity at non-cytotoxic doses in human breast cancer MCF-7 cells (**Figure 7**). Inhibition of NF-κB activation by apples and selected phytochemicals, which are ordinary in the diet, has advantage in cancer therapy when combined with the chemotherapeutic agents. They can be used as a support therapy to lower the cancer cell resistance to the chemotherapy by inhibiting cell proliferation that is activated by NF-κB and by activating apoptosis. Apple extracts and curcumin may provide support therapy in cancer treatment by lowering the toxicity and increasing the efficacy of chemotherapeutic agents. The isolation and identification of active compounds in apple extracts are currently being pursued in our laboratory.

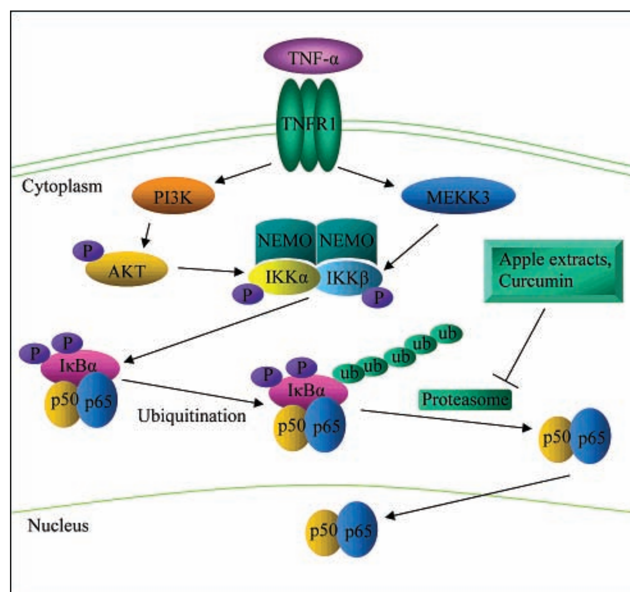


Figure 7. Proposed mechanism of apple extracts and curcumin to inhibit TNF-α-induced NF-κB activation. Apple extracts and curcumin inhibit proteasome that degrades ubquitinated IκB-α to release NF-κB. TNF-α, tumor necrosis factor-α; TNFR1, TNF receptor 1; PI3K, phosphatidylinositol 3′-kinase; MEKK3, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 3; NEMO, NF-κB essential modulator; IκB-α, inhibitor of NF-κB; IKK, IκB-α kinase; P, phosphate; ub, ubiquitin.

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