Inhibition of AP-1 and MAPK signaling and activation of Nrf2/ARE pathway by quercitrin

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Abstract. Quercitrin, glycosylated form of flavonoid compounds, is widely distributed in nature. Extensive studies have demonstrated that quercitrin exhibits strong antioxidant and anti-carcinogenic activities. However, the molecular mechanism is poorly understood. The present study examines the effects of quercitrin on tumor promotion in mouse JB6 cells, a validated model for screening cancer chemopreventive agents and elucidating the molecular mechanisms. Quercitrin blocked TPA-induced neoplastic transformation in JB6 P^+ cells. Pretreatment of JB6 cells with quercitrin down-regulated transactivation of AP-1 and NF-κB induced by UVB or TPA. In the skin of AP-1-luciferase transgenic mice, topical treatment of the mouse with quercitrin markedly blocked the TPA-induced AP-1 transactivation. Further studies indicated that these inhibitory actions appear to be mediated through the inhibition of MAPKs phosphorylation, including ERKs, p38 kinase, and JNKs. In addition, quercitrin stimulated the activation of NF-E2-related factor (Nrf2) and GST ARE-luciferase activity. Comet assays showed that quercitrin could block DNA damage induced by UVB. To our knowledge, these results provide the first evidence that quercitrin contributes to the inhibition of neoplastic transformation by blocking activation of the MAPK pathway and stimulation of cellular protection signaling. Moreover, to our knowledge, these findings provide the first molecular basis for the anti-carcinogenic action of quercitrin.

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Introduction

Previous studies have indicated that reactive oxidative species (ROS) function as second messengers in numerous signaling pathways involved in a diverse array of biological responses ranging from transcriptional regulation, differentiation and proliferation to oncogenic transformation (1,2). Increased formation of ROS can promote the development of malignancy, and the 'normal' rates of ROS generation may account for the increased risk of cancer development and neurodegenerative disorders (3,4). Therefore, interventions favoring the scavenging of ROS (dietary and pharmacological antioxidants) to attenuate the oxidative stress may prevent oxidant stress-associated diseases. A report published by the American Institute for Cancer Research regarding dietary prevention of cancer indicates that 7-31% of all cancers worldwide could be reduced by diets high in fruits and vegetables (5). Thus, searching for novel natural agents and defining novel targets for chemoprevention have become an important area of investigation.

Flavonoids are components of the human diet and are widely found in vegetables and fruits (4). Flavonoids exert various biological activities, which are mainly related to their abilities to inhibit enzymes, to their antioxidant properties, and to their effects on immune responses (6). These activities may explain the beneficial effects that flavonoid intake exerts in different human pathologies, including hypertension, inflammatory conditions, and cancer (7). Quercetin is the most abundant bioflavonoid compound, which is mainly present in the glycoside form. The sugar portion bound to the aglycone portion in quercitrin increases its solubility in polar solvents and consequently improves absorption (8). Recent *in vitro* cell culture or *in vivo* animal experiment studies have demonstrated that quercitrin exhibits a scavenger and anti-oxidant role (9). Therefore, the study of the potential anti-carcinogenic activity of quercitrin has attracted the attention of researchers who intend to identify whether this compound could be an effective chemoprevention and chemotherapeutic agent. Although literature data indicate that quercitrin is a potent antioxidant, the exact mechanism(s) which underlie its protective effects is poorly understood (9).

The cellular signaling cascades mediated by transcription factors, including activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), have been shown to play pivotal roles in

tumor initiation, promotion and progression (10). Activation of AP-1 and/or NF-κB results in the expression of target genes that are involved in many disease processes, such as inflammation, neoplastic transformation, tumor progression, metastasis and angiogenesis (11). Activated AP-1 and NF-κB are found in many different cancer cells (12). The signal transduction pathways of AP-1 and/or NF-κB are known to be important molecular targets of chemo-preventive strategies (13). The components of AP-1 are activated by three distinct but parallel mitogen-activated protein kinases (MAPKs), c-jun NH2-terminal kinase (JNK), p38, and extracellular signal-regulated kinases (ERKs). AP-1, NF-κB, and associated MAPK signal transduction pathways are believed to be crucial in cell transformation and tumor promotion (14). Because of the critical role of AP-1 and NF-κB signaling in inflammation and carcinogenesis, they have been proposed as targets for chemo-preventive agents (13).

Nrf2, a transcription factor, has been shown to play an essential role in the antioxidant response element (ARE)-mediated expression of phase 2 detoxifying enzymes and stress-inducible genes (15). Regulation of both basal and inducible expression of cytoprotective genes is mediated in part by the ARE, a *cis*-acting sequence found in the 5'-flanking region of genes encoding many phase 2 enzymes, including heme oxygenase-1, glutathione S-transferase (GST) A1, NAD(P)H quinone oxidoreductase (NQO1), as well as Nrf2 itself (16). The likely importance of these protective enzymes is highlighted by recent observations that Nrf2-null mice were considerably more sensitive to the tumorigenicity of benzo[a]pyrene (17) and form higher levels of DNA adducts following exposure to carcinogens (18). Thus, the induction of phase 2 gene expression is an effective strategy for achieving protection against carcinogenesis.

Ultraviolet B (UVB), as a tumor initiator, is the most frequently used photocarcinogen in animal studies (19-21). 12-*O*-tetradecanolyphorbol-13-acetate (TPA) is used as a promoter in skin tumor development in mice (21). Our previous studies indicate that both UVB and TPA can activate AP-1 or NF-κB signal pathways (22,23). Because of the important role of AP-1, NF-κB and Nrf2 in carcinogenesis, the present study investigated the effect of quercitrin on UVB- or TPA-induced AP-1-MAPKs and NF-κB activation and Nrf2/ARE activity. Furthermore, the protective effects of quercitrin against UVB-induced DNA damage were also evaluated.

Materials and methods

Materials. Quercitrin and TPA were purchased from Sigma Aldrich (St. Louis, MO). Eagle's minimal essential medium (EMEM) and Dulbecco's modified Eagle's medium (DMEM) were obtained from BioWhittaker Biosciences (Walkersville, MD). Opti-MEM I medium was obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), trypsin, gentamicin and L-glutamine were purchased from Life Technologies, Inc. (Gaithersburg, MD). Luciferase assay kits were purchased from Promega (Madison, WI). Luciferase reporter plasmids for GSTA1 ARE were kindly provided by Drs M.K. Kwak and T.W. Kensler (Johns Hopkins University, Baltimore, MD) (24). FuGENE6 Transfection reagent was purchased from Roche Diagnostics (Mannheim, Germany). Phospho-antibody kits were purchased from Cell Signaling Technology (Beverly, MA). All other antibodies were obtained from Santa Cruz Biotechnology Co. (Santa Cruz, CA). Reagent kit for single cell gel electrophoresis assay was purchased from Trevigen (Gaithersburg, MD). Cell proliferation kit I (MTT) was obtained from Roche Applied Science (Penzberg, Germany).

Cell culture. The JB6 P^+ mouse epidermal cell line and JB6 cells, stably transfected with either AP-1-luciferase or NF-κB-luciferase reporter plasmid (25), were cultured in EMEM containing 5% FBS, 2 mM L-glutamine, and 100 units/ml penicillin and 0.1 mg/ml streptomycin. The cells were grown at 37˚C in a 5% CO_2 atmosphere and 80% humidity.

Total antioxidant capacity assay. The total antioxidant capacity assay was carried out as described previously in JB6 cells (26). Briefly, the total antioxidant capacity of quercitrin was determined using an ABTS (2, 2'-azino-diethylbenz-thiazoline sulfonate) test set (Randox Laboratories Ltd., UK). The principle of the assay depends on production of the radical cation ABTS+ in incubation medium containing the substrates (H_2O_2 and peroxidase), which is a blue-green color and can be detected at 600 nm. The suppression degree of this color production caused by the antioxidants in the sample is proportional to their concentrations. The assays were calibrated against standards and expressed as micromoles per liter.

Comet assay. Comet assay (single-cell gel electrophoresis) was carried out according to the procedure of Sinle *et al* (27,28). Briefly, JB6 cells ($5x10^4$) were seeded in 24-well plates. Six hours later, cells were starved by incubation in 0.1% FBS medium for 12 h. The cells were pretreated with various concentrations of quercitrin for 1 h and then exposed to UVB (4 kJ/m^2) irradiation. All the steps were conducted in dimmed light to prevent additional DNA damage. Comets were visualized using a fluorescence microscope (Olympus, AX70) with an image capture system (SamplePCI, Compix Inc., Cranberry Township, PA). For each sample, 50 cells were scored at magnification, x400. The length of the comet tail, which indicates DNA migration, from the digitized images was determined as the distance between edge of head and end of tail using an image analysis system (Optimas 6.51, Media Cybernetics Inc., Silver Spring, MD) (29).

Cytotoxicity assay. Effects of quercitrin on cell proliferation were evaluated using Electric Cell-substrate Impedance Sensor (ECIS) Model 1600R (Applied BioPhysics, Troy, NY). The ECIS assay has been used for continuous measurement of cell micromotion, attachment, spreading and growth. The JB6 cells (10^4) were suspended in 400 μl of EMEM with or without quercitrin and seeded on electrodes. The cells were equilibrated in the incubator for 15 min. The rate of cell proliferation on the microelectrode was monitored for 72 h as real-time changes in resistance.

Cytotoxicity of quercitrin on JB6 cells was assessed with an MTT assay kit following the manufacturer's instructions. Cells were plated at a density of 10^4 cells/well in a 96-well plate. Cells were cultured, differentiated, and treated with or without quercitrin. Following 72 h incubation, 10 μl MTT labeling reagent was added in each well and the plates were

further incubated for 4 h. Then 100 μl solubilization solution was added, and the plate was incubated overnight at 37˚C. The optical density (OD) of the wells was measured at a wavelength of 590 nm with reference of 650 nm using an ELISA plate reader. Results were calibrated with OD measured without cells in culture.

Assay of AP-1 and NF-κB activity in vitro and in vivo. A confluent monolayer of JB6 cells, stably transfected with either AP-1-luciferase or a NF-κB-luciferase reporter plasmid, was trypsinized, and $2.5x10^4$ viable cells suspended in 0.5 ml of EMEM supplemented with 5% FBS were seeded into each well of a 48-well plate. Plates were incubated overnight at 37˚C in a humidified atmosphere of 5% CO_2. The medium was then switched to 0.1% FBS EMEM, and cells were cultured for 24 h to minimize basal activity of AP-1 or NF-κB. The cells were pretreated with or without quercitrin for 1 h and then exposed to TPA (20 ng/ml) or UVB irradiation (4 kJ/m^2). The cells were extracted with 150 μl of 1X lysis buffer provided in the luciferase assay kit by the manufacturer. Luciferase activity was measured as described previously (22). The results are presented as relative AP-1 or NF-κB activity compared to untreated control.

The *in vivo* effect of quercitrin on the AP-1 activity was explored by using AP-1-luciferase reporter transgenic mice (30,31). The mice were bred and housed in the West Virginia University (WVU) Animal Facility. The animal protocols were approved by WVU-ACUC. Both male and female mice (6-9 weeks old) were used. Dorsal skin of the mice was shaved, and 2 days later a single dose of 1.34 mg quercitrin dissolved in 300 μl of acetone was applied by dermal exposure in the mice. One hour after treatment with quercitrin, the mice (except the negative control group) were treated by dermal exposure to 10.51 μg of TPA dissolved in 300 μl acetone. The negative control mice were treated with acetone only. The luciferase activities were detected by using dorsal skin punch biopsy samples as described previously (30,31). Briefly, the skin tissues were obtained by a 1.5-mm Biopsy Punch (Acuderm Inc., FT. Lauderdale, FL) after exposure. The tissues were lysed in 2X lysis buffer (from the luciferase assay kit) at 4˚C for 12 h. Luciferase activity of AP-1 was measured.

Immunocytochemical staining. Cells were grown on sterilized cover slips until 60-80% confluent and then pretreated with quercitrin. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100/PBS for 30 min, and blocked with 3% bovine serum albumin in PBS for 1 h at 37˚C. The cells were then incubated with NF-κB anti-p65 antibody (sc-372, 1:1000) or anti-AP-1 anti-c-jun antibody (sc-1694, 1:1000) at 37˚C for 3 h. After washing with PBS, Alexa Fluor 488 secondary antibody was used. Fluorescence images were acquired with a confocal laser scanning microscope (Zeiss LSM 510, Thornwood, NY).

Western blot and protein kinase phosphorylation assay. Briefly, JB6 cells were plated onto a 6-well plate. The cultures were grown 24 h and then starved in 0.1% FBS EMEM overnight. Cells were pretreated with different concentrations of quercitrin for 1 h and then were exposed to UVB (4 kJ/m^2, for 14 min) irradiation or TPA (20 ng/ml). After 12 h incubation, the protein was extracted from the cells with 1X SDS sample buffer. Immunoblots for phosphorylation of JNKs, p38, and ERKs were carried out as described in the protocol of the manufacturer. Nonphospho-specific antibodies against JNKs, p38 and ERKs proteins provided in the assay kits were used to normalize the phosphorylation assay, using the same transferred membrane blot (22).

Nuclear translocation assay of Nrf2. Nuclear extracts from JB6 cells were prepared as described previously (32). Briefly, cells were treated in the presence or absence of quercitrin. After treatments, cells were harvested and suspended in hypotonic buffer A [10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF] for 10 min on ice and then vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000 x g for 20 sec and were resuspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The supernatants containing nuclear proteins were collected after centrifugation at 12,000 x g for 2 min. Nrf2 proteins in either nuclear or in whole cell lysate were detected by Western blot analysis. Anti-actin antibody was used as a loading control.

Measurement of GST A1 ARE-luciferase activity. A DNA sequence containing the GST A1 ARE (-833 to -533 from the start codon) was inserted into a luciferase reporter vector as described previously (24). JB6 cells were plated on a 24-well plate at a density of 50-60% confluence. The cells were grown overnight, and the transfection complex containing 0.5 μg plasmid DNA in Opti-MEM I medium was added to each well. Twenty-four hours after transfection, quercitrin was added to cells and then incubated for another 24 h. Luciferase activity was measured using the luciferase assay kit as described above.

Enzyme activity assay. Total GST activity of the cytosolic extracts was measured spectrophotometrically as described previously using chloro-2,4-dinitrobenzene (CDNB) as the substrate (33). JB6 cells seeded in a 24-well plate were incubated with quercitrin for 24 h. The cells were washed and then lysed with 200 μl lysis buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 1% Triton X-100) for 30 min. Cytosolic fractions were prepared by centrifugation at 12,000 x g for 10 min. Assays were conducted in a thermostate compartment at 25˚C. Cytosolic protein (45 μg) was added to 800 μl of reaction mixture containing 100 mM KH_2PO_4 (pH 6.5) and 1 mM glutathione. The reaction was initiated by adding 1 mM CDNB, and the formation of thioether after 5 min was measured at 340 nm. Total enzymatic activity of GST was expressed as nmol/min/mg protein.

For the NQO1 detection, the cells were grown in a 96-well plate, treated with various concentrations of quercitrin, and lysed by 0.8% digitonin. The reaction solution [25 mM Tris-HCl (pH 7.4), 0.06% bovine serum albumin, 5 μM FAD, 1 mM glucose 6-phosphate, 30 μM NADP, 300 units of glucose-6-phosphate dehydrogenase, 725 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and 50 μM menadione]

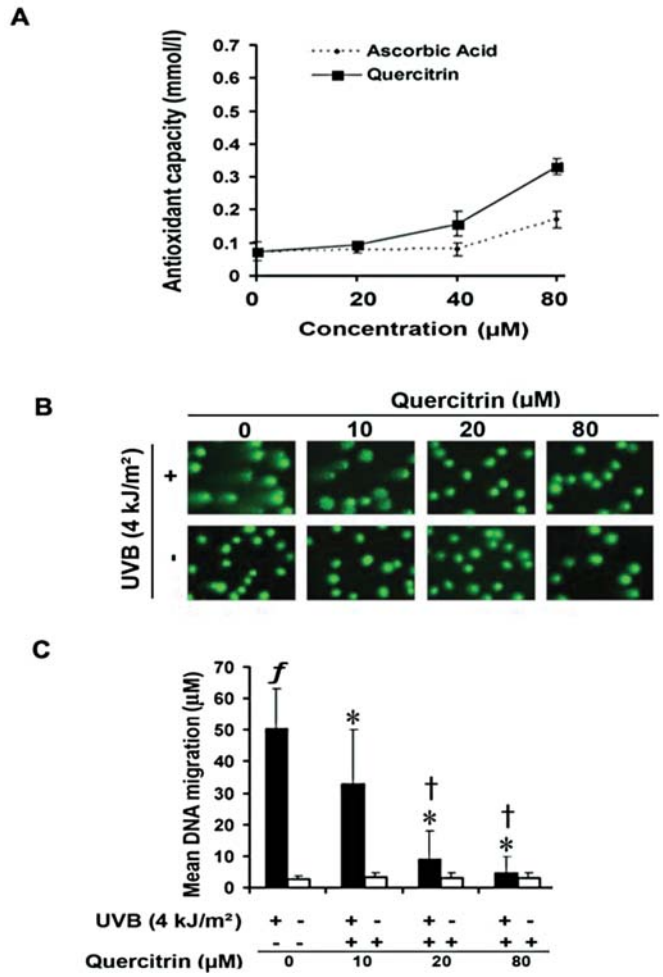

Figure 1. Antioxidant property of quercitrin. (A) The total antioxidant capacity of quercitrin was measured using an ABTS assay. Data shown as total antioxidant capacity are means ± SE of three independent assays. Quercitrin elicited a similar antioxidant activity compared to ascorbic acid over the same concentration range analyzed using pair-wise t-test ($p>0.05$). Comet assay was carried out to detect the antioxidant action of quercitrin on the DNA damage induced by UVB irradiation in JB6 cells. (B) Representative images of cell comet were captured using microscopy at magnification, x400. The lengths of comet tails from digitized images were determined by measuring the distance between edge of head and end of tail, and expressed as DNA migration in microns. (C) Fifty cells were scored for each sample. Data were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks (all Pair-wise Multiple Comparison Procedures, Tukey Test). *Significant decrease of length of the comet tails when quercitrin was added compared to UVB radiation treatment alone ($p\leq0.05$). ^{f}Significant increase of length of the comet tails compared to negative control. †Significant decrease of length of the comet tails compared to treatment group of UVB+10 μM quercitrin.

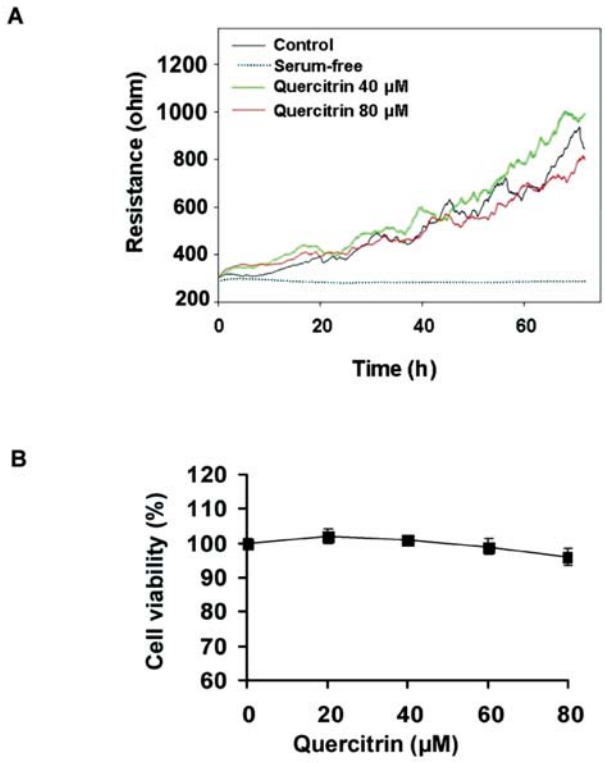

Figure 2. Effect of quercitrin on cell proliferation of A549 and JB6 cells detected by ECIS and MTT assays. (A) A549 cells were monitored in the electrode array wells under the incubation conditions of no treatment, serum free medium, 40 and 80 μM quercitrin for 72 h as real-time changes in resistance. Data shown are representative of two independent experiments. (B) The influence of quercitrin on JB6 cell proliferation was evaluated by the MTT assay following 72 h incubation with various concentrations of quercitrin (0, 20, 40, 60 and 80 μM).

was added into the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was measured at 610 nm. NQO1 induction by quercitrin was expressed as ratios of treated over vehicle control.

Anchorage-independent transformation assay. The effect of quercitrin on TPA-induced cell anchorage-independent transformation was evaluated in JB6 P^+ cells using the soft agar assay as described previously (26). The cells (10^4) were exposed to TPA (20 ng/ml) in the presence or absence of different concentrations of quercitrin, in 1 ml of 0.33% basal medium Eagle agar containing 15% FBS over 3.5 ml of 0.5% agar containing 15% FBS EMEM. The cultures were maintained in a 37˚C, 5% CO_2 incubator for 14 days, and the anchorage-independent colonies were counted as described previously (34).

Statistical analysis. Data are presented as means ± SE of number of experiments/samples as noted in the figure legends. All data were analyzed using SigmaPlot 9.0 software. Significance was set at $p\leq0.05$.

Results

Total antioxidant capacity of quercitrin. Fig. 1A shows quercitrin displayed a similar antioxidant activity compared to ascorbic acid over the same concentration range ($p>0.05$), suggesting that quercitrin can effectively scavenge ROS as well as ascorbic acid.

Inhibition of quercitrin on UVB-induced DNA damage. To investigate the protective action of quercitrin on UVB-induced oxidative damage, the comet assay was carried out in JB6 cells. Cultures of JB6 cells were pretreated with or without quercitrin at a concentration range of 10, 20 and 80 μM for 30 min. Then, cells were subjected to exposure of UVB (4 kJ/m^2) irradiation for 14 min. At 1 h following irradiation, cells were prepared as described in Materials and methods. Comet tails were monitored using a fluorescence microscope

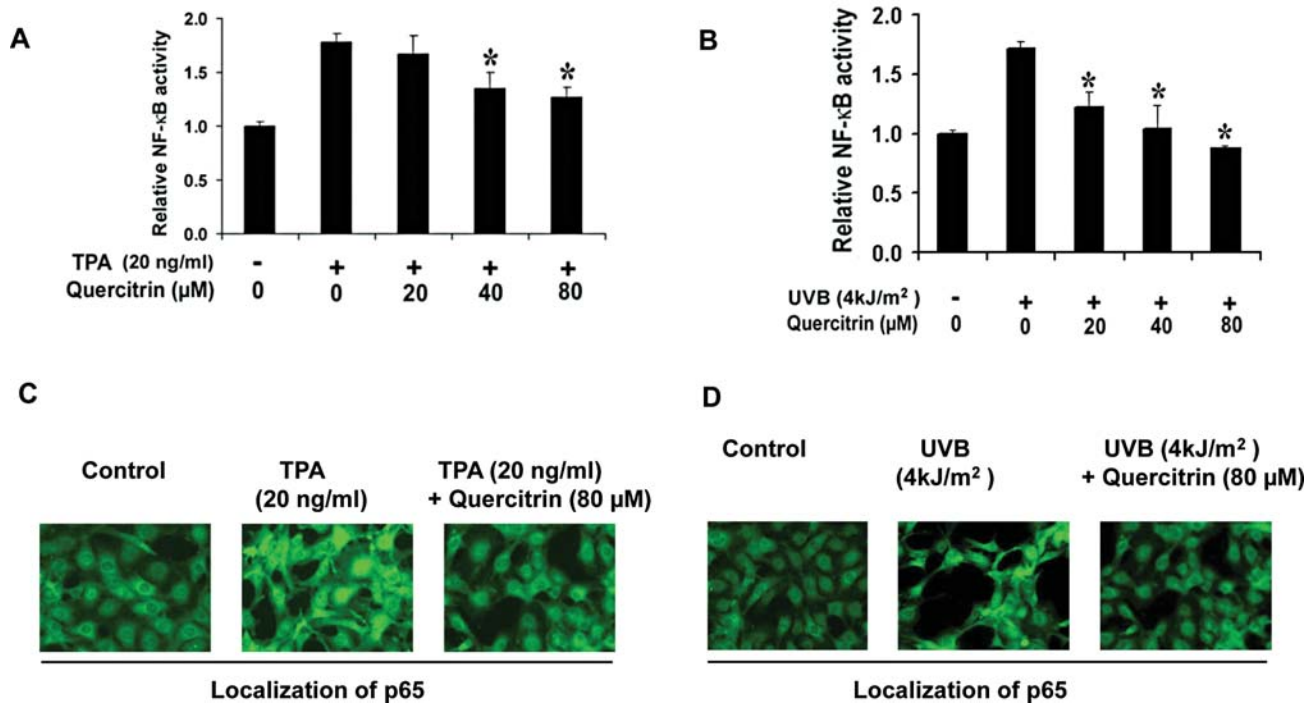

Figure 3. Inhibition of quercitrin on TPA- and UVB-induced NF-κB activation *in vitro*. JB6 cells were pretreated with different concentrations of quercitrin for 1 h and then exposed to 20 ng/ml of TPA or UVB (4 kJ/m^2) irradiation for 14 min (A and B). After culturing the cells at 37˚C for 48 h, the NF-κB activity of the cell extract was measured by the luciferase activity assay. *Significant inhibition of NF-κB activity when quercitrin was added compared to TPA or UVB radiation treatment alone ($p \leq 0.05$) analyzed using t-test (Mann-Whitney Rank Sum Test). Indirect immunofluorescence analysis indicated that pretreatment of the cells with quercitrin significantly blocked TPA- or UVB-induced nuclear translocations of p65 subunit of NF-κB (C and D).

(Fig. 1B). Means of tail length were calculated for each comet, and the mean values for each data set derived. Means of the comet tail length were then plotted against different treatments. The mean of the comet tail length treated with UVB alone was significantly enhanced compared with negative controls. Quercitrin alone, even at a concentration of 80 μM, did not induce a significant increase of tail length, but quercitrin displayed a significant suppression in a dose-dependent manner on UVB irradiation-induced tail length (Fig. 1C).

Effects of quercitrin on cell proliferation and viability. To evaluate the cytotoxicity of quercitrin, ECIS and MTT assays were performed. JB6 cells were monitored on the microelectrode with or without quercitrin for 72 h as real-time changes in resistance by using the ECIS assay. Cells cultured in serum-free medium were used as a negative control. As shown in Fig. 2A, there was no obvious inhibitory effect on proliferation of the cells at 80 μM of quercitrin. Similar results were obtained in the MTT assay (Fig. 2B), at a concentration of 80 μM, quercitrin did not suppress cell proliferation in JB6 cells compared with negative control cells. These results suggest that quercitrin is an effective antioxidant with little cytotoxicity.

Effects of quercitrin on TPA- or UVB-induced NF-κB and AP-1 activation. The effects of quercitrin on TPA- and UVB-induced NF-κB and AP-1 activities were investigated using a reporter gene assay both *in vitro* and *in vivo*. Pretreatment of cells with quercitrin significantly inhibited TPA- and UVB-induced NF-κB activation in a dose-dependent manner (Fig. 3A and B). Indirect immunofluorescence analysis indicated that pretreatment of the cells with quercitrin significantly blocked TPA- and UVB-induced nuclear translocations of p65 subunit of NF-κB (Fig. 3C and D).

The effects of quercitrin on TPA- and UVB-induced AP-1 activation were also tested. The results indicated that pretreatment of the cells with various concentrations of quercitrin produced a dose-dependent decrease in AP-1 activation induced by either TPA or UVB irradiation (Fig. 4A and B). Quercitrin treatment also significantly blocked TPA- and UVB-induced nuclear translocations of the c-jun subunit of AP-1 (Fig. 4C and D).

The *in vivo* effect of quercitrin on TPA-induced AP-1 activity was explored using AP-1-luciferase reporter transgenic mice (Fig. 4E and F). The dorsal skin of the mice was pretreated topically with quercitrin for 30 min and then exposed to TPA. The luciferase activities were measured on dorsal skin punch biopsy samples. Pretreatment of quercitrin significantly suppressed TPA-induced AP-1 activation at 72 h (Fig. 4F).

Effect of quercitrin on TPA- or UVB-induced activation of MAPKs. Fig. 5A and B show that quercitrin suppressed UVB- and TPA-induced phosphorylation of p38, ERK and JNK in a dose-dependent manner. After 24 h treatment, quercitrin induces GST ARE-luciferase activity increasing significantly at 40 and 80 μM (Fig. 5C).

Western blot analysis revealed that the protein levels of Nrf2 in the nucleus were increased following the treatment of cells with 40 μM quercitrin for 3 h, whereas Nrf2 in total cell

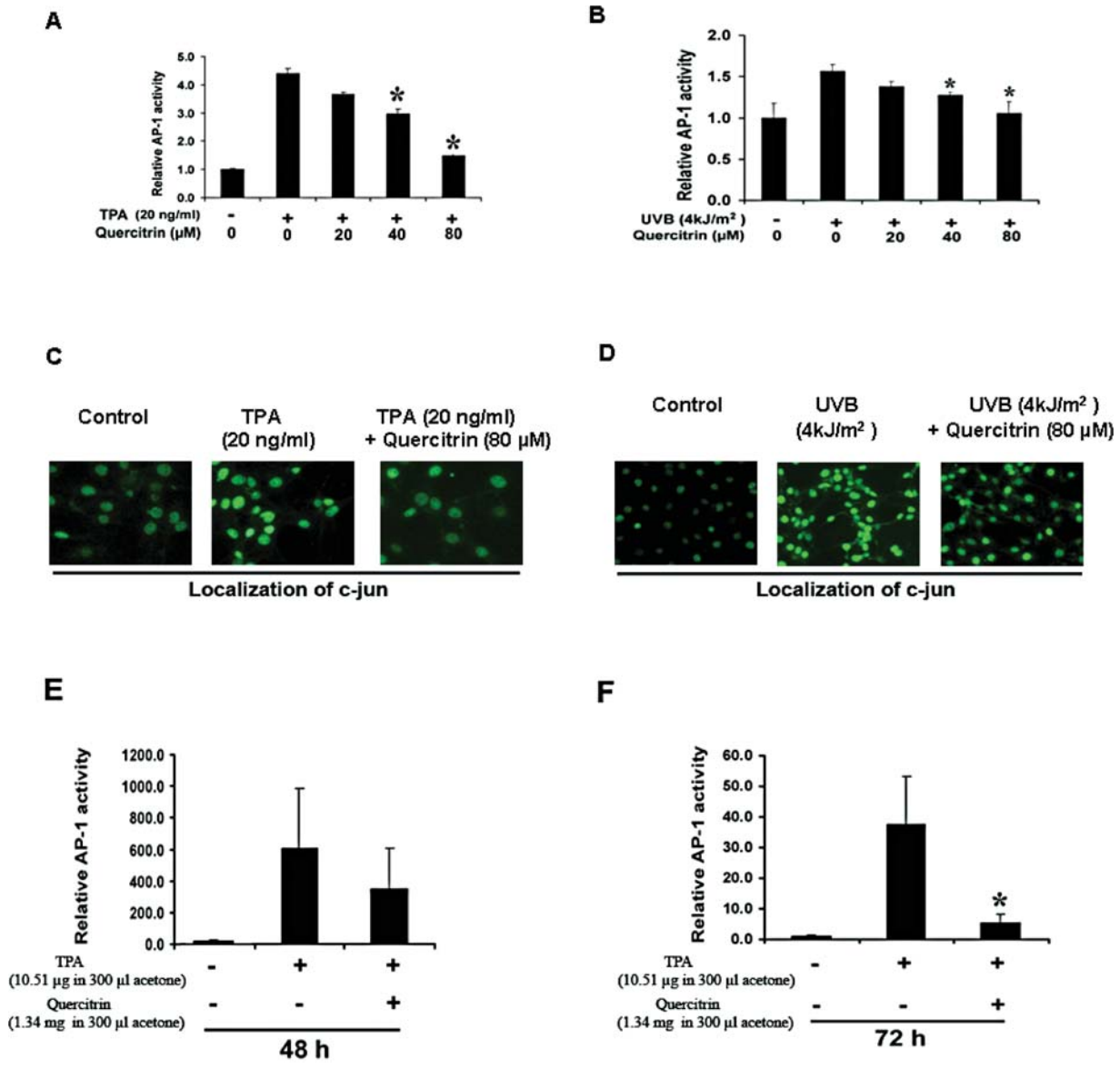

Figure 4. Inhibition of quercitrin on TPA- and/or UVB-induced AP-1 activation *in vitro* or *in vivo*. JB6 cells were pretreated with different concentrations of quercitrin for 1 h and then exposed to 20 ng/ml of TPA or UVB (4 kJ/m^2) irradiation for 14 min (A and B). After culturing the cells at 37˚C for 48 h, the AP-1 activity of the cell extract was measured by the luciferase activity assay. *Significant inhibition of AP-1 activation when quercitrin was added compared to TPA or UVB radiation treatment alone ($p \leq 0.05$) analyzed using t-test (Mann-Whitney Rank Sum Test). Indirect immunofluorescence analysis indicated that pretreatment of the cells with quercitrin significantly blocked TPA- or UVB-induced nuclear translocations of c-jun subunit of AP-1 (C and D). The *in vivo* inhibition of quercitrin on the AP-1 activity induced by TPA was explored by using AP-1-luciferase reporter transgenic mice as described in Materials and methods. At the end of the experiment (48 and 72 h), the skin tissues were obtained by a 1.5-mm biopsy punch (14 mice in control group including 7 males and 7 females, 21 mice in treatment group including 11 males and 10 females). The tissues were lysed in lysis buffer at 4˚C for 12 h. Luciferase activity of AP-1 was measured (E and F). Results, expressed as means ± SE, are representative of two independent experiments. *Significant inhibition of AP-1 activation by quercitrin compared to TPA treatment alone analyzed by using t-test (Mann-Whitney Rank Sum Test) ($p \leq 0.05$).

lysate remained unchanged (Fig. 5D). Moreover, we found that Nrf2 translocation induced by quercitrin was PI 3-kinase-dependent, since LY294002 (an inhibitor of the PI 3-kinase pathway) pretreatment could partially block Nrf2 translocation in JB6 cells (Fig. 5D).

Quercitrin stimulates GST and NQO1 enzymatic activity in JB6 cells. To evaluate the possibility that quercitrin possesses a similar induction of detoxifying enzymes, JB6 cells were incubated with quercitrin for 24 h, and the NQO1 and total GST activities were measured. Quercitrin significantly stimulated both GST and NQO1 enzymatic activity in a dose-dependent manner (Fig. 6A and B). At concentrations of 40 and 80 μM of quercitrin, GST and NQO1 activities were increased by about 100%, respectively.

Quercitrin inhibits TPA-induced JB6 cell transformation. Previous studies have demonstrated that AP-1 and/or NF-κB activation is required in neoplastic transformation and tumorigenesis in JB6 P$^+$ cells (35). We thus tested the effect of quercitrin on TPA-induced cell transformation using the soft agar assay. Results shown in Fig. 6C indicate that TPA-induced

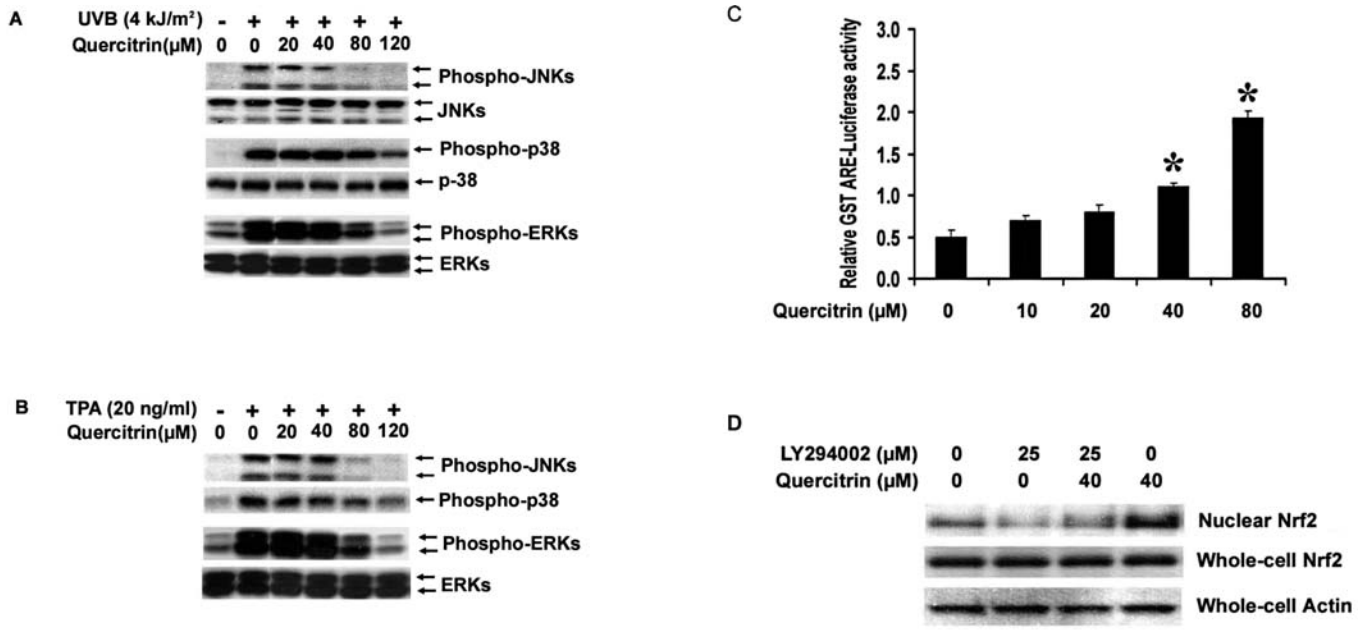

Figure 5. Effects of quercitrin on phosphorylation of MAPKs, GST A1 activation, and Nrf2 nucleus translocation. JB6 cells were pretreated with different concentrations of quercitrin for 1 h and then were exposed to UVB (4 kJ/m^2, for 14 min) irradiation or TPA (20 ng/ml). After 12 h incubation, the phosphorylated and non-phosphorylated JNK, p38 and ERK proteins in the cell lysate were assayed. The phosphorylated proteins and nonphosphorylated proteins were detected, using the same transferred membrane blot following a stripping procedure. The inhibitory effects of quercitrin on UVB- (A) or TPA (B)-induced phosphorylation of JNK, p38 and ERK were detected by Western blot analysis. GST A1 activation induced by quercitrin was conducted with various concentrations of quercitrin for 24 h, and the luciferase activity was measured (C). Results are expressed as means ± SE of three assay wells. The experiment was repeated three times. *Significant increase in ARE-luciferase activity induced by quercitrin compared with control cells ($p \leq 0.05$) analyzed using t-test (Mann-Whitney Rank Sum Test). JB6 cells either with or without LY294002 pretreatment for 30 min was used in the experiment for the detection of Nrf2 expression. Then, cells were incubated with 40 μM quercitrin for 3 h. Nrf2 protein level in the nucleus or in total cell lysate was detected by Western blot analysis (D).

cell transformation was significantly inhibited by quercitrin at concentrations of 40 and 80 μM.

Discussion

Chemoprevention, the use of drugs or natural substances to inhibit carcinogenesis, is an important and rapidly evolving aspect of cancer research. It provides a practical approach to identifying potentially useful inhibitors of cancer development. Quercitrin, the glycoside form of quercetin, is a bioflavonoid with antioxidant properties, and exerts anti-inflammatory and antioxidant activities demonstrated by *in vitro* cell cultures or *in vivo* animal models (7,9). This study reports molecular evidence for anti-carcinogenic potential for quercitrin. The results from this study suggest that quercitrin displays a similar antioxidant activity as well as ascorbic acid. Quercitrin also inhibited TPA-induced neoplastic transformation and prevented DNA damage induced by UVB irradiation. The mechanistic studies suggest that the possible anti-carcinogenic activity may be due to the inhibitory effects on NF-κB and AP-1-MAPKs signaling and the inductive effects on phase 2 gene activities.

ROS have been known to be associated with many diseases. ROS are associated not only with initiation, but also with promotion and progression in the multistage carcinogenesis mode (36). It has been reported that oxidative stress induces activation of transcription factors and the expression of several oncogenes, including AP-1, NF-κB, c-*myc*, and c-*fos*, which enhance cell proliferation (37). Reducing oxidative stress may suppress the proliferation of tumor cells (38). The results of the current study demonstrate that quercitrin can act as an antioxidant as well as ascorbic acid, over the same concentration range *in vitro*. This suggests that the chemopreventive activity of quercitrin may be due to its antioxidant properties. It has been shown that UVB radiation produces DNA damage directly and indirectly through oxidative stress (39). UVB irradiation, which stimulates ROS generation and causes DNA damage, is known to evoke the emergence of apoptotic cells (40). In this study, the antioxidant properties of quercitrin may contribute to its protective effect against UVB-induced DNA damage.

AP-1 and NF-κB are transcription factors that have been implicated in a wide range of cell biological events, including cell proliferation, inflammation, differentiation, metastasis, and apoptosis. On the other hand, inhibition of AP-1 and NF-κB activations by a variety of agents has been shown to reduce neoplastic transformation (25). *In vivo* studies in transgenic mice indicate that AP-1 transactivation is required for tumor promotion (25). The blockade of TPA-induced cell transformation by quercitrin might be through the inhibition of AP-1 and NF-κB activity. Therefore, the inhibitory effects of quercitrin on AP-1 and NF-κB activation noted in this study suggest a potential beneficial role in preventing carcinogenesis.

Although many mechanisms may be involved in the up- and down-regulation of AP-1 activity, MAPKs including ERKs, JNKs and p38 kinases are known to be common signaling pathways mediating AP-1 activity (41). Activation of the MAPK pathway is a frequent event in tumorigenesis. MAPKs are activated in response to environmental stresses and growth factors. JNK, p38, and ERK are key molecules

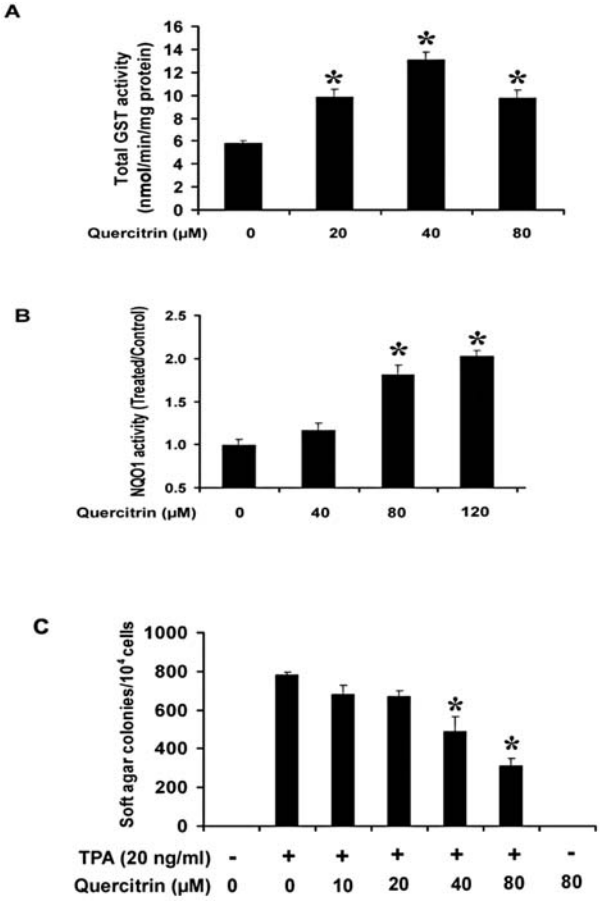

Figure 6. Quercitrin stimulates GST and NQO1 activity and inhibits TPA-induced JB6 cell transformation. JB6 cells were incubated with quercitrin for 24 h. Total GST and NQO1 activity was detected. Total GST activity was expressed as nmol/min/mg protein (A). For NQO1 activity, the values are means ± SE and are expressed as ratios of treated over vehicle control (B). The experiment was repeated three times. *Significant induction of total GST or NQO1 activity by quercitrin compared to controls ($p \leq 0.05$) analyzed using t-test (Mann-Whitney Rank Sum Test). The effect of quercitrin on TPA-induced cell anchorage-independent transformation was determined as described under Materials and methods. The cell colonies were scored by a computerized image analyzer. The values (mean ± SE) shown are a representative of two independent experiments performed in triplicate (C). *Significant decrease of soft agar colony formation when quercitrin was added compared to TPA treatment alone ($p \leq 0.05$) analyzed using t-test (Mann-Whitney Rank Sum Test).

activated in response to oxidant injury (42,43). Previous experiments have shown that both UVB and TPA can induce ROS generation in the cells (44,45). In this study, we found that quercitrin could inhibit both UVB- and TPA-induced phosphorylation of JNK, p38 and ERKs. These observations suggest that the inhibitory effects on AP-1 and MAPKs activation with quercitrin may be due partially to its antioxidant properties.

The capacity of cells to maintain homeostasis during oxidative stress resides in activation or induction of protective enzymes. Among them, the induction of detoxification and antioxidant enzymes by natural and synthetic chemopreventive agents serves as a major protective mechanism against carcinogenesis. As a member of bZIP transcription factors, Nrf2 is expressed in a variety of tissues and plays an important role in regulating the expression of these mammalian detoxifying and antioxidant enzymes under oxidative stress. Transcriptional activation of antioxidant genes through an antioxidant response element (ARE) is largely dependent upon Nrf2 (46). The present study indicates that quercitrin could induce GST and NQO1 enzymatic activities, Nrf2 nuclear translocation, as well as GSTA1 ARE-luciferase activation. These results indicate that a possible mechanism of the antioxidant actions of quercitrin may be via induction of phase 2 detoxifying enzymes, such as GST and NQO1.

In conclusion, we have demonstrated that the quercitrin exhibits antioxidant activities in both *in vitro* and *in vivo* experiments, which is possibly achieved by the activation of Nrf2 that is critical for the expression of phase 2 antioxidative enzymes. The antioxidant activities of quercitrin may further prevent the activation of MAPKs, AP-1 and NF-κB. These results suggest that quercitrin may function as a potential anticancer agent with little cytotoxicity to normal cells. Thus, quercitrin merits further investigation as a cancer therapeutic and preventive agent in humans. These studies establish a promising area of investigation in understanding the molecular mechanisms responsible for the beneficial effects of polyphenols on human health.

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