The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells


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Dietary phytochemicals have been shown to be protective against various types of cancers. However, the precise underlying protective mechanisms are poorly understood. In the present study, we report that treatment of A549 cells with quercetin resulted in a dose-dependent reduction in cell viability and DNA synthesis with the rate of apoptosis equivalent to 1.2 ± 0.8, 6.3 ± 0.9, 16.5 ± 1.5, 36.4 ± 2.6 and 42.5 ± 5.8% on treatment with 0.1% dimethylsulfoxide, 14.5, 29.0, 43.5 and 58.0 μM quercetin, respectively. Concomitantly, quercetin treatments led to a 1.1-, 1.1-, 2.5- and 3.5-fold increase in Bax. Similar elevations were also observed in Bad, which increased 1.1-, 2.1-, 2.2- and 2.3-fold, respectively, as compared with control. While Bcl-2 was decreased by 30%, Bcl-xL was elevated in a dose-dependent fashion. Quercetin also induced the cleavage of caspase-3, caspase-7 and PARP (poly ADP-ribose polymerase). While Akt-1 and phosphorylated Akt-1 were inhibited, the extracellular signal-regulated kinase (ERK) was phosphorylated following quercetin treatment in a dose-dependent fashion. Phosphorylation of ERK and c-Jun occurred at 3 h and was sustained over 14 h. Phosphorylation of MEK1/2 increased in concordance with ERK activation. Quercetin-induced phosphorylation of c-Jun N-terminal kinase (JNK) and cleavage of caspase-3 occurred 6 h after quercetin exposure and before cleavage of caspase-7 and PARP was detected. Inhibition of MEK1/2 but not PI-3 kinase, p38 kinase or JNK abolished quercetin-induced phosphorylation of c-Jun, cleavage of caspase-3 and -7, cleavage of PARP and apoptosis. Inhibition of caspase activation completely blocked quercetin-induced apoptosis. Expression of constitutively activated MEK1 in A549 cells led to activation of caspase-3 and apoptosis. The results suggest that in addition to inactivation of Akt-1 and alteration in the expression of the Bcl-2 family of proteins, activation of MEK-ERK is required for quercetin-induced apoptosis in A549 lung carcinoma cells.

Introduction

Lung cancer is one of the most common cancers in many countries and accounts for 28% of all cancer death (reviewed in refs 1,2). Approximately 75–85% of lung cancer is non-small cell lung cancer and the rest is small cell lung cancer (3). The average 5-year survival rate for localized lung cancer was 48% compared with 14.5% overall and 2.5% for a metastatic lung cancer. Five-year survival rate after surgical resection was ~60% for stage I lung cancer patients (4). Unfortunately, only 15% of people are diagnosed at an early, localized stage because most lung cancer begins to grow silently without any symptoms until the cancer is in an advanced stage (5). Thus, there is an urgent need for novel diagnosis, prevention and/or treatment of lung cancer.

Epidemiological studies have shown that the consumption of vegetables, fruits and tea is associated with a low risk of cancer (6). Many natural dietary phytochemicals found in fruits, vegetables, spices and tea have been shown to be protective against cancer in various animal models (7,8). The most common flavonoid glycones found in the diet are quercetin, rutin and robinin (9). Although the mechanisms by which quercetin exerts its anti-proliferative and apoptotic activities remain to be elucidated, there is evidence suggesting that the action of flavonoids is probably mediated by either interaction with the type II estrogen binding sites (10) or aryl hydrocarbon receptor (11). Quercetin has been shown to inhibit the enzymes involved in proliferation and signal transduction pathway including protein kinase C (12), tyrosine kinase (13), cdc25 phosphatase (14), PI-3 kinase (15), DNA topoisomerase II (16), prolinc-directed protein kinase fatty acid in human prostate carcinoma cells (17), Na+K+ATPase (18) and c-Jun N-terminal kinase (JNK) (19). Quercetin has a wide range of biological activities including inhibition of mutant p53 expression (20), and androgen receptor expression and function in LnCap cells (21). It has anti-proliferative activity in vitro against several cancer cells of human origin (22–26). It blocks the cell cycle at the G1/S transition in colon and gastric cancer as well as in leukemic cells (27,28) but causes a G2/M block in breast and laryngeal cancer cell lines or in non-oncogenic fibroblasts (20,29,30). Quercetin potentiates the cytotoxic action of 1-β-d-arabinofuranosylcytosine (31). Quercetin also inhibits cell invasion and induces apoptosis through a pathway involving heat shock proteins (32). These activities of quercetin make it a promising candidate for treatment and prevention of various cancers.

One of the most frequent targets downstream of receptor and non-receptor tyrosine kinases and the ras family of GTP-binding proteins is the extracellular-related kinase (ERK) signal transduction pathway (33). Constitutive MEK1 activation contributes to cell survival (33), migration (34), transformation of fibroblasts and epithelial cells (35). Studies with small molecule inhibitors of MEK activity (36) demonstrate a role for MEK in mediating expression of proteinases implicated in invasion and metastasis (37), and disruption of normal epithelial morphology (38). Treatment of cells with various growth factors or chemotherapeutic agents produces activation of MEK1/2 and its downstream target, ERK, resulting in

Abbreviations: DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly ADP-ribose polymerase; SRF, serum-free RPMI 1640; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.
proliferation and differentiation (reviewed in ref. 33). ERK activation may exert either anti-apoptotic (reviewed in ref. 33) or pro-apoptotic (39) influence depending upon the cellular context.

Regulation of apoptosis is a complex process and involves a number of cellular genes, including Bcl-2 (40) and Bcl-2-related family members such as Bcl-xL, Bcl-xS, Bad and Bax (41). The Bcl-2 gene product protects cells against apoptosis in a variety of experimental systems. Suppression of Bcl-2 has been shown to promote apoptosis in response to a number of stimuli, including anticancer drugs (40). Bcl-2 and Bcl-xL exert their anti-apoptotic effect, at least in part by binding to Bax and related pro-apoptotic proteins. They also prevent Bax and pro-apoptotic proteins from inducing the release of cytochrome c and activation of the caspase-9. Recent work into apoptosis has demonstrated the importance of PI-3 kinase and its downstream substrate protein kinase B (Akt) (42). Akt exerts an anti-apoptosis effect against various stimuli (42) and confers resistance to taxol (43). A direct link between the PI-3 kinase and apoptosis-regulating proteins was established through Akt phosphorylation of Bad (44, 45).

We demonstrate herein that quercetin inhibited the A549 cell growth and induced apoptosis. Quercetin-induced apoptosis was associated with inactivation of Akt-1 protein, altered expression of Bcl-2 family of proteins and phosphorylation of ERK. Our studies, employing transfection study and pharmacological inhibitors for MEK-ERK and caspase revealed that MEK-ERK activation was required to trigger caspase-3 activation and that MEK-ERK blockade modified the cytotoxicity of quercetin, indicating that MEK-ERK activation in A549 cells is linked to cell death.

Materials and methods

Reagents

U0126, PD98059, LY294002, rabbit anti-phospho MEK1/2 (Ser217/221), rabbit anti-cleaved caspase-7 (20 kDa), rabbit anti-caspase-3, rabbit anti-phospho Akt (Ser473), mouse anti-phospho p44/42 ERK (Thr202/Tyr204), rabbit anti-Akt, mouse anti-ERK and rabbit anti-cleaved poly ADP-ribose polymerase (PARP) antibodies were purchased from New England Biolabs, Beverly, MA. Caspase inhibitor Z-VAD-FMK was from Promega, Madison, WI. A c-Jun N-terminal kinase specific inhibitor SP600125, a p38 kinase inhibitor SB203580 and G418 were supplied by Calbiochem, La Jolla, CA. Caspase inhibitor Z-VAD-FMK and G418 were supplied by Promega, Madison, WI. Horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit antibodies were purchased from Jackson ImmunoResearch, West Grove, PA. Tissue culture Petri dishes, 6-well plates, 96-well plates, 100 mm tissue culture Petri dish, and 8-chamber slides were purchased from Nunc, Naperville, IL. Quercetin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, St. Louis, MO. Cell Proliferation ELISA Kit (BrdU, colorimetric assay) and In Situ Cell Death Detection Kit (Fluorescein) were supplied from Roche Diagnostics Corporation, Indianapolis, IN. RPMI 1640 medium, fetal bovine serum (FBS), Lipofectamine reagent and penicillin-streptomycin were from Gibco BRL, Grand Island, NY. U0126, PD09859, SP600125, SB203580, LY294002 and caspase inhibitor Z-VAD-FMK compounds were dissolved in dimethylsulfoxide (DMSO) with final concentration never exceeding 0.1%. Quercetin was dissolved in DMSO at a concentration of 100 mg/ml. They were stored frozen under light-protected conditions at −20 °C.

Cell culture and treatment

Human A549 lung epithelial cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin (growth medium) at 37 °C in a 5% CO2 incubator. It has been documented that A549 cells are pRB-positive (46) and express wild-type p53 (47). To study the effects of quercetin on cell morphology, A549 cells were seeded onto 6-well plates at a density of 5 × 10^4 cells/well in the growth medium. Confluent cells were washed with serum-free RPMI 1640 (SRF) medium and then incubated further with SRF medium for 6 h. Cells were then treated with 14.5, 29.0 and 58.0 μM of quercetin in SRF medium for 14 h. Photographs were taken using the inverse microscope (Nikon TMS, Tokyo, Japan).

Cell transfection

To examine whether constitutively activated MEK1 had any impact on the survival of lung cancer cells, A549 cells were transfected with 10 μg of the pβ-MEKDD or pcDNA-3 control plasmid DNA and 28 μl of Lipofectamine reagent following the manufacturer’s recommendation. The pβ-MEKDD was the constitutively activated MEK1 cDNA, kindly provided by Professor Phillip Leder (48). Forty-eight hours post-transfection, cells were subcultured at a ratio of 1:10 and replaced with selective growth medium containing 800 μg/ml G418. Twenty weeks post-transfection, pooled clones were expanded. To study the effects activated MEK1 on quercetin-induced apoptosis, constitutively activated MEK1-transfected or pcDNA-transfected cells were seeded at a density of 1 × 10^4 cells/100 mm Petri dish and allowed to grow in growth medium for 24 h. Cells were treated with indicated concentrations of quercetin in serum-free RPMI for 14 h and then harvested for analysis.

Detection of apoptosis

A549 cells were plated onto 8-chamber slides at a density of 5 × 10^4 cells/well and allowed to grow in the growth medium for 24 h. Cells were then washed once with SRF medium and treated with indicated concentrations of quercetin in fresh SRF medium for 24 h. Cells were fixed with phosphate buffer saline (PBS) containing 4% formaldehyde for 1 h at room temperature, washed with PBS and stored at −80 °C until analysis. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) as described by the manufacturer. Slides were visualized under fluorescent microscope (Olympus BX60) equipped with an FITC filter. Labelling indices were obtained by counting cell number of labelled cells among at least 500 cells/region and expressed as percentage values.

Cell viability and proliferation

To study the effects of quercetin on cell proliferation and viability, A549 cells were plated at 1 × 10^5 cells/well in 96-well plates and allowed to grow in the growth medium for 24 h. Cells were then washed once with SRF medium and allowed to grow in SRF medium for another 24 h. Cells were then treated with indicated concentrations of quercetin in fresh SRF medium for 24 or 48 h. Cell proliferation and cell viability were determined at 24 and 48 h after treatment using the Cell Proliferation ELISA Kit and the MTT assay respectively as described (49). Experiments were repeated at least three times, and the data were expressed as the mean ± SD.

Western blot analysis

To examine the effects of quercetin on Bax, Bad, Bcl-2, Bcl-xL, cleaved caspase-3 and -7, cleaved PARP, PI-3 kinase, Akt and phosphorylation of ERK, c-Jun, JNK and Akt, A549 cells were plated at a density of 5 × 10^5 cells/100 mm Petri dish in the growth media. After 24 h, the cell monolayer was washed and then treated with indicated concentrations of quercetin in SRF medium as described above. Following the treatment, cells were harvested at indicated times, lysed in a lysis buffer and western blot analysis was performed using total cell lysate as described (50). For each lane 100 μg of protein were loaded. Blots were incubated with the indicated antibodies and 1:7500 horse-radish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibody. All the primary antibodies were used at the final concentration of 1 μg/ml. Blots were then visualized with a chemiluminescent detection system as described by the manufacturer.

Statistical analysis

For quantification analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated, and normalized to the amount of α-tubulin. After normalization with α-tubulin, changes in the expression of the protein under study in treated samples were expressed relative to the basal levels of this protein in untreated sample. Differences in cell proliferation, apoptosis and the levels of proteins under study were analysed by the Kruskal-Wallis test.

Results

For the time-course and dose-response experiments, human A549 lung cancer cells were treated with 14.5, 29.0, 43.5 and 58.0 μM of quercetin for 24 and 48 h. Cell viability and cell death
growth were assessed by the MTT assay and BrdU incorporation, respectively. Control cells were treated with 0.1% DMSO alone. Figure 1 shows that quercetin caused a dose-dependent reduction in DNA synthesis (Figure 1A) and cell viability (Figure 1B). Significant inhibition in BrdU incorporation was observed as early as 24 h post-treatment ($P < 0.01$). Fifty percent reduction in cell viability was seen at a dose of 29.0 μM after 48 h incubation (Figure 1B). To determine if quercetin reduced cell number by inducing apoptosis, TUNEL assay was performed. Figure 2A shows that DNA fragmentation was detected in quercetin-treated cells. In cells treated with 0.1% DMSO, 14.5, 29.0, 43.5 and 58.0 μM of quercetin for 24 h, approximately 1.2 ± 0.8, 6.3 ± 0.9, 16.5 ± 1.5, 36.4 ± 2.6 and 42.5 ± 5.8% of apoptotic cells were observed, respectively (Figure 2B). The percentage of apoptotic cells in the treatment groups compared with the control group were statistically significant at $P < 0.01$ as determined by the Kruskal-Wallis test.

Because apoptosis in mammalian cells has been shown to be regulated by Bax, Bcl-X<sub>L</sub>, Bad, Bcl-2 (41), we determined whether quercetin-induced apoptosis in A549 cells was also associated with the modulation of these proteins. To test this possibility, cell lysate from A549 cells treated with different concentrations of quercetin was examined by western blot analysis. As shown in Figure 3, quercetin induced a significant elevation in the expression of pro-apoptotic Bax and Bad. Treatment of A549 cells with 14.5, 29.0, 43.5 and 58.0 μM of quercetin for 14 h led to 1.1-, 1.1-, 2.5- and 3.5-fold increase in Bax. Similar elevations were observed in Bad levels, which increased 1.1-, 2.1-, 2.2- and 2.3-fold, respectively, in the quercetin treatment as compared with controls. While Bcl-2 expression was decreased by ~30%, the anti-apoptotic Bcl-X<sub>L</sub> expression was increased by 2.0-, 2.3-, 2.4- and 2.5-fold on treatment with 14.5, 29.0, 43.5 and 58.0 μM of quercetin, respectively (Figure 3D). Because phosphorylation of Bad at Serine 112 and 136 created consensus sites for interaction with 14-3-3 protein; phosphorylated Bad then bound to 14-3-3 instead of Bcl-2 or Bcl-X<sub>L</sub>, resulting in the liberation of the anti-apoptotic proteins and the consequent

![Fig. 1. Effects of quercetin on the viability and proliferation of A549 cells. A549 lung cancer cells were grown and treated with SRF medium containing either 0.1% DMSO or indicated doses of quercetin for 24 and 48 h as described under Materials and methods. Cell proliferation (A) and cell viability (B) were determined by Bromo-uridine incorporation and MTT assay respectively as described under Materials and methods. Experiments were performed in quadruplicate, with the results reflecting the mean and standard deviation of the quadruplicate of each group. Bars with different letters are significantly different from one another at $P < 0.01$ as determined by the Kruskal-Wallis test. The experiments were repeated three times with similar results.](https://academic.oup.com/carcin/article-lookup/25/5/647/2390638)
promotion of cell survival (51), we determined the phosphorylation status of Bad following quercetin treatment. Using anti-phospho-specific Bad (Ser112) and Bad (Ser136) antibodies, we observed that Bad was not phosphorylated at these positions (data not shown). The results indicate that quercetin-induced apoptosis in A549 cells is associated with the accumulation of Bax, Bad, Bcl-xL and the decrease in Bcl-2 proteins.

It has been demonstrated that the proteolytic cleavage of PARP, which synthesizes poly(ADP-ribo) from β-nicotinamide adenine dinucleotide (NDA) in response to DNA strand breaks, is a biochemical event during apoptosis (52). As PARP cleavage is a hallmark of caspase activation, we determined whether the apoptosis machinery was activated by quercetin treatment, using an anti-specific-cleaved PARP antibody that detects only cleaved products of PARP. As shown in Figure 4E, the 89 kDa cleaved PARP fragment was detected in quercetin-treated samples. As caspase-3 and -7 have a central role in PARP cleavage (52), we determined the activation of these two caspases by western blot analysis using antibodies capable of detecting activated (cleaved) caspase-3 and -7. Figure 4C shows that the cleaved forms of caspase-3 (19 and 17 kDa fragments) were readily detectable at the dose as low as 14.5 μM of quercetin and reached high levels at the dose of 29.0 μM. Cleaved caspase-7 fragments (19 and 20 kDa) were also observed in concordance with

Fig. 2. Induction of apoptosis by quercetin in A549 cells. A549 cells were grown and treated with escalating doses of quercetin in SRF medium for 14 h. Apoptotic cells were determined by TUNEL assay as described in Materials and methods. (A) Apoptotic cells were visualized under a fluorescent microscope. The rate of apoptosis expressed as percentage of total cells counted is shown in (B). Bars with different letters are significantly different from one another at $P < 0.01$ as determined by the Kruskal-Wallis test. Experiments were repeated three times with similar results.
Fig. 3. Effects of quercetin on the levels of Bcl-2, Bax, Bad and Bcl-xL in A549 cells. A549 cells were cultured as described under Materials and methods. Cells were treated with 0.1% DMSO or indicated concentrations of quercetin in SRF medium for 14 h. Cells were harvested and lysed for western blot analysis as described under Materials and methods. Blots were incubated with mouse anti-α-tubulin (A), mouse anti-Bax (B), rabbit anti-Bad (C), rabbit anti-Bcl-xL (D) and rabbit anti-Bcl-2 (E) antibodies. Changes in the levels of Bax, Bad, Bcl-2 and Bcl-xL proteins after being normalized to the levels of α-tubulin are shown below each blot. Experiments were repeated three times with similar results.

Fig. 4. Effects of quercetin on cleavage of caspase-3 and -7 and PARP in A549 cells. A549 cells were cultured as described under Materials and methods. Cells were treated with SRF medium containing 0.1% DMSO or indicated concentrations of quercetin for 14 h. Cells were harvested and lysed for western blot analysis as described under Materials and methods. Blots were incubated with mouse anti-α-tubulin (A), rabbit anti-cleaved caspase-3 (B), rabbit anti-cleaved caspase-7 (20 kDa) (C) and rabbit anti-cleaved PARP (D) antibodies. Changes in the levels of cleaved caspase-3, cleaved caspase-7 and cleaved PARP after being normalized to the levels of α-tubulin are shown below each blot. Experiments were repeated three times with similar results.
caspase-3 activation (Figure 4D). The data indicate that quercetin induced the activation of caspase-3 and -7.

PI-3 kinase pathway is regulated by a variety of growth factors (43,44). Recent work on apoptosis signalling has demonstrated the importance of PI-3 kinase and its downstream substrate, Akt (42,53). In addition, a direct link between PI-3 kinase and apoptosis-regulating protein Bcl-2 family of proteins has been established through Akt phosphorylation of Bad (44,45). We determined the effects of quercetin on the levels of p85 subunit of PI-3 kinase, Akt-1 and phosphorylated Akt (Ser473) in A549 cells. Figure 5B shows that the expression of p85 subunit of PI-3 kinase was not altered following quercetin treatment. However, treatment of A549 cells with 14.5, 29.0, 43.5 and 58.0 μM of quercetin for 14 h resulted in a 30, 44, 48 and 58% decrease in total Akt protein (Figure 5D). The basal phosphorylation of Akt-1 was sharply reduced and barely detectable in cells treated with 29.0 μM of quercetin (Figure 5C). The results indicate that quercetin was more effective in inhibiting the phosphorylation of Akt-1 than Akt-1 expression. Subsequent blotting with anti-α-tubulin antibody showed relatively equal amounts of total protein loaded per lane.

It has been demonstrated that the ERK signalling pathway is activated in response to certain cellular stresses (reviewed in ref. 54). To investigate whether quercetin induced growth arrest and apoptosis in the A549 cell was associated with the activation of ERK, cell lysate from quercetin-treated cells at different times were subjected to western blot analysis using an anti-phospho-ERK antibody to detect phosphorylated (and therefore activated) ERK. The same blots were subsequently stripped and rebotted with an antibody that recognized total ERK to verify equal amounts of the protein in various samples. As shown in Figure 6, treatment of A549 cell with 14.5, 29.0, 43.5 and 58.0 μM of quercetin, all of which induced apoptosis, led to a dose-dependent activation of ERK (Figure 6D). The phosphorylation of MEK1/2 was increased in a dose-dependent manner following quercetin treatment over the same time frame as seen for ERK (Figure 6B). Because c-Jun is a target for ERK activity, the levels of c-Jun phosphorylation were determined. Figure 6F shows that c-Jun was phosphorylated following quercetin treatment and the pattern of c-Jun phosphorylation was similar to ERK activation suggesting that phosphorylation of ERK by MEK1/2 increased ERK activity. As JNK activation is thought to be a determining factor in cell cycle arrest and apoptosis (55), we set out to determine whether quercetin-induced apoptosis in A549 lung cancer cells was also associated with the activation of JNK/SAPK pathway. The activation of JNK was determined by examining its degree of phosphorylation by western blot analysis with anti-phospho-JNK1/2 antibody. Figure 6G shows that quercetin significantly enhanced the phosphorylation of a 54 kDa JNK2 without significant increase in the basal phosphorylation of 46 kDa JNK1. Blocking of JNK activity with JNK inhibitor SP600125 did not prevent quercetin-induced apoptosis (Figure 9) suggesting that JNK is not a mediator of quercetin-induced apoptosis.

To study the time-course of MEK1/2, ERK, c-Jun, caspase-3, -7 and JNK activation, cells were treated with 0.1% DMSO or 58.0 μM of quercetin and then harvested at different times for western blot analysis. As shown in Figure 7D, activation of ERK was detected as early as 3 h, reached maximum levels 6 h after quercetin treatment and sustained over the 14 h period (Figure 7D). The result was corroborated by demonstrating that ERK kinase activity was increased by 4-fold 6 h after quercetin treatment as determined by the levels of c-Jun phosphorylation (Figure 7F). MEK1/2 was also phosphorylated by quercetin treatment over the same time frame as seen for ERK (Figure 7B). Phosphorylation of the 54 kDa JNK2 was detectable 6 h after quercetin exposure (Figure 7G). Cleaved forms of caspase-3 and -7 were clearly detected 9 h after quercetin treatment and became more pronounced at 14 h (Figure 7H and I). Significant PARP (89 kDa) cleavage was detected 14 h after quercetin treatment (Figure 7K).

It has been demonstrated that ERK activation may exert pro-apoptotic influence depending upon the cellular context.
To determine whether quercetin-induced apoptosis is mediated by the activation of ERK, two approaches were used. The first strategy was to treat the cells with pharmacologic blockers of MEK1/2 to inhibit quercetin-induced ERK activation and their downstream effects. The second approach was to express the constitutively activated MEK1 in these cells. Quercetin, in combination with MEK1/2 inhibitors U0126 and PD98059, or a PI-3 kinase inhibitor LY294002, or p38 kinase inhibitor SB203580, or JNK inhibitor SP600125 or caspase inhibitor Z-VAD-FMK were also used to treat human A549 lung carcinoma cells. The apoptosis was measured by TUNEL assay. The levels of cleaved caspase-3, cleaved caspase-7 and cleaved PARP were also investigated. TUNEL assay showed that quercetin and combined quercetin-LY294002 caused apoptosis in A549 cells (Figure 8D and F). Co-treatment of A549 cells with quercetin and U0126 or PD98059 completely blocked quercetin-induced apoptosis as determined by TUNEL assay (Figure 8E). Western blot analysis revealed that quercetin alone significantly increased phosphorylation of ERK, c-Jun, cleaved PARP, cleaved caspase-3 and cleaved caspase-7 (Figure 9). Like quercetin, both LY294002 and U0126 were capable of inducing 54 kDa JNK2 phosphorylation. Unlike U0126 and PD98059, LY294002 alone caused a mild increase in cleaved PARP, cleaved caspase-7, cleaved caspase-3 (Figure 9) and apoptosis (Figure 8C). Co-treatment of cells with quercetin and U0126 or PD98059 prevented quercetin-induced phosphorylation of ERK, phosphorylation of c-Jun, cleavage of caspase-3 and -7 and cleavage of PARP (Figure 9). Quercetin-induced 54 kDa JNK2 phosphorylation was also attenuated by U0126-quercetin or PD98059-quercetin treatment (Figure 9E). Blocking PI-3 kinase, p38 kinase and JNK activity neither potentiated nor prevented quercetin-induced apoptosis, cleavage of caspase-3 and -7 and cleavage of PARP (Figure 9). Inhibition of caspase activation by Z-VAD-FMK abolished quercetin-induced cleavage of caspase-3 and PARP and apoptosis (Figure 9). The results suggest that activation of MEK-ERK plays an important role in quercetin-induced apoptosis and ERK acts upstream of caspase-3 and -7 to exert its apoptotic influence in the quercetin-treated A549 cells.
Since the blocking of MEK activity by chemical inhibitors prevented quercetin-induced apoptosis in A549 cells, we wished to determine if constitutive activation of MEK1 in A549 cells would make the cells more sensitive to quercetin-induced apoptosis. A549 cells were transfected with pcDNA-3 or constitutively activated MEK1 (48). After 3 weeks of selection in G418, pooled mock-transfected and constitutively activated MEK1-transfected cells were treated with 0.1% DMSO or 58 μM of quercetin for 14 h and harvested for analysis. Because cleaved PARP by activated caspase-3 and -7 has been proposed as one of the events in the execution phase of apoptosis, the levels of cleaved caspase-3, -7 and PARP were used as markers for apoptosis. As shown in Figure 10, cleaved caspase-3, -7 and PARP were detected in activated MEK1-transfected cells grown in serum free medium. In contrast, these apoptotic markers were not detected in mock-transfected cells in the absence of quercetin. Furthermore, activated MEK1-transfected cells were more susceptible to quercetin-induced apoptosis than mock-transfected cells as determined by the increased levels of apoptotic markers and TUNEL assay (Figure 10G). The results further support the important role of activated MEK-ERK in quercetin-induced apoptosis in A549 cells.

Discussion

The relationship between diet and cancers has been implicated in several epidemiological studies (6). The cancer incidence is significantly lower in people whom diet consists largely of fruits and vegetables than people whom diet consists mainly of animal products (6). The results from several studies indicate that vegetables and fruits contain components that have anti-proliferative and anti-neoplastic properties (56,57). In the present study, we have shown that quercetin inhibits human A549 lung cancer cell proliferation and induces apoptosis. TUNEL assay clearly shows DNA fragmentation. In addition to the inhibition of Akt-1 phosphorylation, alteration in the expression of Bcl-2 family of proteins, sustained activation of ERK is required for quercetin-induced apoptosis in A549 cells. Quercetin treatment results in dose- and time-dependent activation of ERK. The elevated ERK activity contributed to apoptosis by quercetin is supported by the observations that activation of ERK by expression of activated MEK1 induces apoptosis while inhibition of ERK by MEK inhibitors blocks quercetin-induced cell death. Quercetin-induced apoptosis is associated with phosphorylation of c-Jun, PARP cleavage, caspase-3 and -7 cleavage, all of which can be reduced by treatment of A549 cells with the MEK1/2 inhibitor. Our findings suggest that in addition to the inhibition of Akt activation and alteration in the ratio between the anti-apoptotic and pro-apoptotic proteins, ERK activation plays an important role in mediating quercetin-induced apoptosis in A549 cells and ERK functions upstream of the caspase activation to initiate the apoptotic signal.

After ingestion, the major circulating metabolites in rat blood are quercetin glycosides, quercetin glucoronides and quercetin sulfates. It remains to be determined whether
Quercetin is metabolized to more or less active metabolites. Our preliminary data indicate that the concentration of quercetin and its metabolites was 25.1, 43.3 and 54.3 μM after 10 days of treatment with 50, 100 and 150 μg of quercetin/kg body wt, respectively (our unpublished data). The results suggest that the concentrations of quercetin employed in the present study can be achieved in vivo.

Two major distinct apoptotic pathways have been described for mammalian cells. One involves caspase-8, which is recruited by the adapter molecule Fas/APO-1 associated death domain protein to death receptors upon extracellular ligand binding (58). We do not observe any change in either Fas or FasL expression in quercetin-treated A549 cells (data not shown). Administration of quercetin results in a significant increase in the expression of Bax and Bad. Furthermore, the levels of Bcl-xL are elevated while the expression of Bcl-2 protein is reduced by quercetin. Overall, there is a shift in the ratio between the anti-apoptotic and pro-apoptotic proteins following quercetin treatment. Although the contribution of the Bcl-2 family of proteins to quercetin-induced apoptosis is not examined in the present study, it is possible that the reduction in Bcl-2 by quercetin allows less Bcl-2–Bax complex. Furthermore, inactivation of Akt prevents Akt-1 from phosphorylating Bad on the serine 112 and serine 136. As a result, Bad becomes bound to Bcl-2, and its pro-apoptotic activity is effectively increased from the death-regulation equation. Elevation of non-phosphorylated Bad by quercetin further helps to sequester more Bcl-2 and Bcl-xL. The net effect is more free Bax, which then translocates to the mitochondrial membrane and induces the opening of the mitochondrial permeability transition pore, a critical event in the loss of cell viability (59).

Fig. 8. Effects of MEK1/2 inhibitor U0126 and PI-3 kinase inhibitor LY294002 on quercetin-induced apoptosis in A549 cells. A549 lung cancer cells were grown and treated with SRF medium containing 0.1% DMSO (A), 10.0 μM of U0126 (B), 10.0 μM of LY294002 (C), 58.0 μM of quercetin (D), 58.0 μM of quercetin plus 10.0 μM of U0126 (E) and 58.0 μM of quercetin plus 10.0 μM of LY294002 (F) for 14 h. Cells were subjected to TUNEL assay as described under Materials and methods. Apoptotic cells were visualized under a fluorescent microscope. Original magnification, ×200.
Anti-apoptotic effects of PI-3K are due to its activation of serine/threonine protein kinase Akt. This kinase has been shown to block apoptosis via several mechanisms (60). In the present report, we observe that quercetin inhibits Akt and Akt phosphorylation. Because Akt is a downstream target of PI-3 kinase, the observed inhibition of Akt phosphorylation suggests that quercetin also inhibits PI-3 kinase. This argument is supported by previous studies showing that quercetin is an inhibitor of PI-3 kinase and serine/threonine protein kinases (12,13,15,19). By suppressing the activation of Akt-1, quercetin can promote apoptosis via several pathways. Inactivation of Akt would prevent Akt-1 from phosphorylating Bad on the serine 136. As a result, Bad becomes bound to Bcl-2, and its pro-apoptotic activity is effectively increased in the death-regulation equation.

Although the effects of quercetin on the gene encoding A1/Bf11 protein is not examined in the present study, it is possible that inhibition of Akt-1 phosphorylation by quercetin might impair the ability of Akt to transactivate the gene encoding A1/Bf11 protein which, under certain experimental conditions, is essential for the release of cytochrome c and/or AIF from the mitochondria (61).

Previous studies indicate that JNK/SAPK activation is associated with apoptosis (55,62). In our own study using A549 lung cancer epithelial cells, we also find that 54 kDa JNK2 takes place in the absence and in the presence of mild apoptosis, respectively. Furthermore, inhibition of JNK activity by SP600125 did not prevent quercetin-induced apoptosis. Therefore, it is unlikely that activation of JNK plays an important role in quercetin-induced apoptosis of A549 cells.

In the present study, we have provided the evidence that activation of MEK-ERK plays a dominant role in quercetin-induced activation of caspase-3 and -7, which is necessary for cleavage of PARP and apoptosis in A549 lung cancer cells. Quercetin treatment results in high and sustained activation of ERK in A549 cells. One important difference between the quercetin and IGF-I induced ERK activation is the time and duration of activity (data not shown). In the case of IGF-I, ERK activation is rapid, occurring within minutes of treatment, and transient. With quercetin, significant activation occurs at 3 h, but the activity remains highly elevated throughout the experiment (up to 14 h). Utilizing U0126 or PD98059 to modulate ERK activity, we find that inhibition of MEK-ERK pathway by taxol delayed or failed to prevent taxol-induced apoptosis (63,64). However, it is not a universal feature of mammalian cells as activation of MEK-ERK pathway has been shown to contribute to cell proliferation and survival (33), migration (34) and transformation (35). Furthermore, inhibition of stress-induced signalling via the MEK-ERK pathway can potentiate the toxic effects of chemotherapeutic drugs and irradiation (65). Therefore, the ability of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phospho-ERK</th>
<th>Phospho-c-Jun</th>
<th>Phospho-JNK-1</th>
<th>Phospho-JNK-2</th>
<th>Cleaved caspase -3</th>
<th>Cleaved caspase -7</th>
<th>Cleaved PARP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin (58 μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>U0126 (10 μM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>LY294002 (10 μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Z-VAD-FMK (50 μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>PD98059 (50 μM)</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>SP600125 (10 μM)</td>
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<td>+</td>
</tr>
<tr>
<td>SB203580 (10 μM)</td>
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</table>

Fig. 9. Effects of MEK1/2 inhibitor U0126 or PD98059, p38 kinase inhibitor SB203580, JNK inhibitor SP600125, caspase inhibitor Z-VAD-FMK and PI-3 kinase inhibitor LY294002 on quercetin-induced phosphorylation of ERK, c-Jun, JNK, and cleavage of caspase-3, -7 and PARP in A549 cells. A549 lung cancer cells were grown and treated with SRF medium containing 0.1% DMSO and indicated concentration of drugs for 14 h. Cells were harvested and lysed for western blot analysis as described under Materials and methods. Blots were incubated with mouse anti-a-tubulin (A), mouse anti-phospho p44/42 ERK (Thr202/Tyr204) (B), rabbit anti-ERK (C), mouse phospho c-Jun (Ser63) (D), mouse anti-phospho JNK (Thr183/Tyr185) (E), rabbit anti-cleaved caspase-3 (F), rabbit anti-cleaved caspase-7 (G) and rabbit anti-cleaved PARP (H) antibodies. Experiments were repeated three times with similar results.
MEK-ERK pathway to regulate proliferation versus survival appears to depend on cell types and the amplitude and duration of ERK activation. A short activation of MEK-ERK cascade by growth factors such as IGF-I is associated with proliferation while prolonged activation of ERK activity inhibits DNA synthesis.

The questions remaining are: how does quercetin induce apoptosis of A549 cells and what are the mechanisms responsible for transmitting the signal to the cell nucleus. It has been proposed that the anti-proliferative and apoptotic effects of quercetin are also mediated via non-estrogen receptor regulated mechanisms (20,29,30). Although the precise mechanisms of the anti-proliferation and apoptosis of quercetin are unknown, there is evidence suggesting that the action of flavonoids is probably mediated by interaction with the aryl hydrocarbon receptor (11) or type II estrogen binding sites (10), which have been shown to occupy by a flavonoid-like molecule with growth inhibitory properties (66). In addition to activation of MEK-ERK as we report in this study, quercetin is shown to induce apoptosis through a pathway involving heat shock proteins (32). Other effects of quercetin may also contribute to its anti-proliferative activity seen in certain experimental systems including inhibition of mutant p53 expression in breast cancer cells (20) and blockage of the cell cycle at the G1/S or G2/M transition (27,28). Quercetin also inhibits various enzymes involved in proliferation and apoptosis including protein kinase C (12), tyrosine kinase (13), cdc25 phosphatase (14), PI-3 kinase (12,15) and DNA topoisomerase II (16). This suggests that quercetin exerts multiple effects on cellular growth and apoptosis. The target proteins observed under one experimental condition or cell type may differ from one another depending on cell context.

Our data support a role for ERK activation in quercetin-induced growth inhibition and apoptosis in lung cancer cells. As quercetin can act in synergy with cisplatin to inhibit lung cancer xenograft growth (67) and potentiate the cytotoxic action of 1-D-arabinofuranosylcytosine (31), it raises the possibility that quercetin can be used either as a single agent or in combination with other chemotherapeutic agents such as cisplatin or 1-D-arabinofuranosylcytosine in treatment and/or prevention of lung cancer.

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References


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Quercetin induces apoptosis in A549 cells

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