The Chemopreventive Bioflavonoid Apigenin Modulates Signal Transduction Pathways in Keratinocyte and Colon Carcinoma Cell Lines$^{1,2}$

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ABSTRACT Apigenin is a nonmutagenic chemopreventive agent found in fruits and green vegetables. In this study, we used two different epithelial cell lines (308 mouse keratinocytes and HCT116 colon carcinoma cells) to determine the effect of apigenin on the mitogen-activated protein kinase (MAPK) cascade. Apigenin induced a dose-dependent phosphorylation of both extracellular signal-regulated protein kinase (ERK) and p38 kinase but had little effect on the phosphorylation of c-jun amino terminal kinase (JNK). We used immunoprecipitation-coupled kinase assays to show that apigenin increased the kinase activity of ERK and p38 but not JNK. Consistent with these results, we found that apigenin induced a 7.4-fold induction in the phosphorylation of Elk, the downstream phosphorylation target of ERK kinase. Similarly, apigenin induced a 3.2-fold induction in the phosphorylation of activating transcription factor-2, the downstream phosphorylation target of p38 kinase. Little change was observed in the phosphorylation of c-jun, the phosphorylation target of JNK. These data suggest that part of the chemopreventive activity of apigenin may be mediated by its ability to modulate the MAPK cascade. J. Nutr. 133: 3800S–3804S, 2003.

KEY WORDS: • flavonoid • apigenin • mitogen-activated protein kinase

Apigenin is a nonmutagenic, naturally occurring flavonoid present in a variety of fruits and leafy vegetables (1,2). A large body of evidence suggests that apigenin shows promise as a chemopreventive agent because it produces a wide variety of effects in cultured cells including inhibition of transformation and angiogenesis (3–5), induction of cell cycle arrest (6), and stimulation of gap junctional intercellular communication (7).

Previous studies by Birt and co-workers demonstrated that topical application of apigenin to mouse epidermis resulted in decreased ornithine decarboxylase activity (8) and a reduction in size of skin tumors induced by chemical carcinogens (3,8) or UV irradiation (9). We confirmed that apigenin inhibited UVB-induced skin carcinogenesis in SKH1 hairless mice even when applied topically several weeks after UVB-irradiation treatments had commenced (unpublished results). Our laboratory is presently investigating the various pathways targeted by apigenin in epidermal keratinocytes and other epithelial cell lines to identify its mechanism of action as a chemopreventive agent.

A number of recent reports demonstrated the differential effects of flavonoids as either inducers or inhibitors of signal transduction pathways involving tyrosine protein kinases and serine/threonine protein kinases (10,11). In the rat pheochromocytoma cell line PC12, apigenin sustained the epidermal growth factor (EGF)$^4$ stimulation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) activation while blocking EGF-induced protein kinase B activation (12). In contrast, apigenin was reported to inhibit ERK1/2 activation in rapidly proliferating cells (4) and in thyroid cancer cells (13). Apigenin and another polyphenolic compound, LY294002, were both shown to compete for the ATP-binding domain as inhibitors of phosphoinositide 3-kinase (14,15). Genistein, an isoconformer of apigenin, was reported by Akiyama and co-workers (16) to be a specific inhibitor of tyrosine protein kinases.

As a result of their effects on regulation of mitogen-activated protein kinase (MAPK) signaling cascades, a number of phytochemicals were shown to subsequently affect the activity of various transcription factors at the endpoints of the MAPK

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$^4$ Abbreviations used: ATF-2, activating transcription factor-2; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated protein kinase 1 and 2; GST, glutathione S-transferase; JNK, c-jun amino terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TLB, Triton lysis buffer.

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pathways. For example, flavone, apigenin, kaempferide, and chalcone were reported to stimulate activator protein 1 activity in human endometrial adenocarcinoma cells and human embryonic kidney 293 cells (11). Apigenin stimulated transcriptional activation of CCAAT/enhancer-binding protein homologous protein but inhibited c-jun activation by phorbol myristoyl acetate (11). In rat aortic vascular smooth muscle cells, apigenin inhibited serum-induced phosphorylation of ERK1/2 and expression of c-fos mRNA, a downstream target of the ERK pathway (17). These results collectively demonstrate that flavonoids possess differential abilities to regulate the MAPK-responsive pathways and subsequent transcriptional activation of signal transduction target genes in mammalian cells.

In this study we investigated the ability of apigenin to modulate cell-signaling pathways involved in the MAPK signal transduction network. We used two epithelial cell lines, the mouse keratinocyte 308 cell line (18) and the HCT116 human colon carcinoma cell line (19), to investigate the effect of apigenin on the three major MAPK pathways: the ERK pathway, the c-jun amino terminal kinase (JNK) pathway, and the p38 pathway. We also investigated the effect of apigenin treatment on representative transcription factors for each of the three MAPK pathways.

MATERIALS AND METHODS

Chemicals and reagents. Apigenin (4′,5,7-trihydroxyflavone, 95% purity) and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Apigenin was prepared in DMSO and added to the medium so that the final concentration of solvent did not exceed 0.1%. Erk, JNK, p38, c-jun, activating transcription factor-2 (ATF-2), Elk, and phosphospecific Erk antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific p38, JNK, c-jun, ATF-2, and Elk antibody were from Cell Signaling Technology (Beverly, MA).

Cell culture. The 308 mouse keratinocyte cell line (18) was cultured in Suspension Minimal Essential Medium (Sigma) supplemented with 8% chexeld (Bio-Rad Laboratories, Hercules, CA) fetal calf serum and 0.05 mmol/L CaCl2. When cultures reached ~90% confluence, cells were treated with adding apigenin in DMSO or DMSO (as vehicle control) to the culture medium. HCT116 p53+/+ cells were a kind gift from B. Vogelstein (Johns Hopkins University, Baltimore, MD) and were cultured in McCoy's 5A medium (Mediatech, Herndon, VA).

Western blot analysis. Cells were harvested at the indicated times in triton lysis buffer [TLB; 1 mmol/L Tris, 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 0.01 μmol/L leupeptin, 0.002 μmol/L aprotinin, 0.2 mmol/L benzamidine, and 6.5 mmol/L dithiothreitol (DTT)]. Protein concentrations were determined using BCA reagent (Pierce, Rockford, IL). Proteins were resolved on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels followed by electrophoretic transfer onto polyvinylidene difluoride membranes. The membranes were blocked using 5% nonfat dry milk. Primary antibody directed toward unmodified proteins were incubated in 5% nonfat dry milk. Western blot analysis was achieved using enhanced chemiluminescence (Amersham, Piscataway, NJ). Enhanced chemiluminescence (Amersham, Piscataway, NJ). Western blot analysis was achieved using enhanced chemiluminescence (Amersham, Piscataway, NJ).

Immune complex kinase assays. After treatment, cells were harvested in TLB subsequent to a 4-h incubation. Aliquots (200 μg) of protein extract were immune precipitated using 3 μL of the appropriate protein A/G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA)–bound antibody per experimental sample. Kinase assays were carried out in buffer containing 25 mmol/L Heps, 25 mmol/L β-glycerophosphate, 0.1 mmol/L sodium vanadate, 0.5 mmol/L ATP, 5 μmol/L DTT, 5 μmol/L ATP, 333 kBq γ-32P ATP, and 8 μg glutathione S-transferase (GST)-tagged c-jun or myelin basic protein (MBP) as substrate. Kinase reactions were carried out at room temperature for 15 min for JNK assays and for 30 min at 30°C for p38 and Erk kinase assays. Reactions were terminated by the addition of SDS sample loading dye and band intensities were quantitated by phosphoimager analysis.

Nuclear fractionation. Nuclear extracts were prepared as described previously (20) with minor modifications. Cells were washed twice in ice-cold phosphate buffered saline (PBS), scraped into 1 mL PBS, and pelleted at 134 × g for 5 min. The cell pellets were washed again in PBS and resuspended in two packed cell volumes of buffer A [10 mmol/L HEPES, pH 8.0, 0.5% (v/v) Nonident P-40, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, and 200 mmol/L sucrose] for 5 min on ice. The nuclear and cytoplasmatic fractions were separated by centrifugation at 15,000 × g for 10 s. The cytoplasmatic fraction was removed and the nuclear pellet washed twice in buffer B (20 mmol/L HEPES, pH 7.9; 1.5 mmol/L MgCl2, 420 mmol/L NaCl, 0.2 mmol/L EDTA, and 1.0 mmol/L DTT) and incubated on ice for 30 min. Nuclei were clarified by centrifugation (15,000 × g) and the supernatant was diluted 1:1 in buffer C [20 mmol/L HEPES, 100 mmol/L KCl, 0.2 mmol/L EDTA, 20% (v/v) glycerol, and 1 mM DTT].

RESULTS

To investigate the effect of apigenin (Fig. 1) treatment on the MAPK cascade, we used the mouse keratinocyte cell line 308. At near confluence, 308 cells were treated with DMSO (0.1% final concentration) or apigenin in increasing concentrations (Fig. 2). At low doses of apigenin (10–30 μmol/L), the phosphorylation of ERK was inhibited slightly, whereas at doses of 50–100 μmol/L, apigenin induced the phosphorylation of ERK kinase. Apigenin treatment also resulted in a dose-dependent increase in the phosphorylation of p38. Apigenin slightly inhibited the phosphorylation of JNK at doses ranging from 30 to 100 μmol/L. Western blot analysis of actin protein levels show that an equal amount of protein was loaded in each gel lane. To determine when apigenin induced the highest level of phosphorylation of p38 and ERK, 308 cells were treated with DMSO or apigenin for 2–10 h and cell lysates were analyzed by Western blot (Fig. 3A). These data show that apigenin induced ERK kinase activity maximally at 4 h, as demonstrated by a 4.6-fold induction in the phosphorylation of ERK. Apigenin also induced a 4.4-fold increase in the phosphorylation of p38 at 4 h and the induction decreased slowly for an additional 4 h. Phosphorylation of JNK was not detectable within this time period (data not shown). Total p38 and ERK levels were measured by Western blot analysis to ensure that the increase in phosphorylation of these proteins was not due to an increase in the total levels of the protein.
To determine whether apigenin-induced phosphorylation of ERK and p38 was cell line specific to the 308 cell line, we carried out time course experiments on the human colon carcinoma cell line HCT116 (Fig. 3B). Similar to results obtained in 308 cells, apigenin induced the phosphorylation of ERK kinase in a time-dependent fashion. A 15-fold induction in phosphorylation of ERK was seen at 2 h and activation continued up to 6 h and then diminished. p38 kinase was also phosphorylated maximally at 2 h, as demonstrated by a 4.3-fold induction in phosphorylation. JNK phosphorylation was not detectable during this period of activation (data not shown).

To establish whether the apigenin-induced increase in phosphorylation of MAPK translated into an increase in its kinase activity, we performed immune complex kinase assays. Cultures of 308 keratinocytes were treated with medium containing apigenin or an equivalent concentration of DMSO as a vehicle control. Cells were lysed in TLB and aliquots of lysates were immunoprecipitated with antibody directed toward ERK, p38, or JNK and included in kinase assays containing MBP (ERK and p38) or c-jun (JNK) as substrate (Fig. 4). In comparison with DMSO-treated cells, apigenin treatment resulted in a 1.8-fold increase in the kinase activity of ERK. A 2.6-fold increase in the kinase activity of p38 was seen in the presence of apigenin. In contrast, 308 cells treated with apigenin contained slightly less JNK activity than did cells treated with DMSO-containing medium.

To determine whether apigenin-induced activation of ERK and p38 kinase resulted in the activation of the downstream target proteins that are transcription factors, nuclear fractions of DMSO- or apigenin-treated cells were isolated according to standard procedures (20) and analyzed by Western blot analysis (Fig. 5). Western blot analysis of nuclear fractions showed that apigenin induced a 7.4-fold induction in the phosphorylation of Elk, a known target of ERK kinase. In addition, a 3.2-fold increase in the phosphorylation of ATF-2 (phosphorylation target of p38) resulted from cell treatment with apigenin. In contrast, the level of phosphorylation of c-jun did not change significantly after 308 cells were treated with apigenin. Western blot analysis of the total protein levels of Elk, ATF-2, and c-jun, was carried out to verify that differences in phospho-Elk and phospho-ATF-2 levels were not due to changes in total Elk and ATF-2 protein levels in the treated cells. Collectively, these data show that apigenin induced the activation of ERK and p38 kinase but had little effect on the activation of JNK.

**DISCUSSION**

A large body of evidence now indicates that the bioflavonoid class of phytochemicals has significant potential as chemopreventive agents against a number of different types of cancer. Numerous reports identified and characterized a wide variety of effects in mammalian cells and tissue attributed to bioflavonoids, yet the specific molecular mechanisms behind the chemopreventive effects exhibited by these compounds remains largely unknown.

Previous studies by our laboratory and others demonstrated that topical application of apigenin inhibited UV-induced tumors in mice in vivo (8,9; Pelling et al., unpublished results, 2003). We previously showed that apigenin treatment induced G2/M arrest in 308 mouse keratinocytes (6) and that this arrest partially resulted from apigenin-induced inhibition of cyclin B1–associated Cdc2p34 kinase activity. Additional studies performed in our laboratory showed that apigenin induced the stabilization and transcriptional activity of the p53 tumor suppressor protein in mouse 308 keratinocytes (21). In this report, we used the same keratinocyte cell line to characterize
the effect of apigenin treatment on modulation of signal transduction pathways and the ensuing effect on downstream targets represented by selected transcription factors. Our experiments showed that apigenin treatment of mouse keratinocytes and human colon carcinoma cells results in activation of the ERK1/2 and p38 MAPK pathways whereas there was little effect on the JNK pathway. We also demonstrated that apigenin treatment of cells significantly increases downstream transcriptional activation factors of the ERK1/2 and p38 MAPK pathways, specifically the Elk transcription factor and the ATF-2 transcription factor, respectively. Consistent with the fact that we did not observe any significant activation of the JNK pathway was our observation that no change in level of phosphorylation of c-jun protein occurred in the treated cells. Our results indicated that apigenin’s mechanism of action in chemoprevention of skin cancer may involve modulation of at least two of the three major MAPK pathways.

A number of other reports described the diverse effects of apigenin treatment on a number of MAPK pathways. Both activation and inhibition of MAPK were attributed to apigenin, and this may in part reflect a cell specificity effect (11–13). In the rat pheochromocytoma cell line PC12, apigenin was found to sustain the activation of ERK1/2 phosphorylation induced by EGF stimulation (12), whereas in thyroid carcinoma cells, apigenin inhibited EGF-receptor tyrosine phosphorylation and activation of ERK1/2 (13). These latter results are in contrast to our findings presented here for epithelial keratinocyte and colon carcinoma cell lines. These different outcomes may be due to differences in other genes in the cells reflecting their tumor origin and multiple genetic events that led to their outgrowth as tumor cell lines.

In this report, we further characterized the molecular targets of the chemopreventive bioflavonoid apigenin. Our results, when taken together with previously published studies, indicated that apigenin exerts its effects on a number of cellular pathways critical to processes involving cell cycle regulation, tumor suppressor protein function, and signal transduction pathways. These studies supported the notion that apigenin may possess a broad spectrum of activity to produce a chemopreventive effect by affecting a number of molecular targets that influence multiple pathways in the cell.

LITERATURE CITED


