Flavonoid Phytochemicals Regulate Activator Protein-1 Signal Transduction Pathways in Endometrial and Kidney Stable Cell Lines[1]

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ABSTRACT Phytochemicals bind to and regulate the human estrogen receptors (ERα and ERβ), mimicking actions of the endogenous estrogen, 17β-estradiol, and known antiestrogens such as ICI 182,780. Recently, however, some of these estrogenic phytochemicals have been shown to affect other signal transduction pathways, such as receptor tyrosine kinases and mitogen-activated protein kinases (MAPK). Previously, we found that certain phytochemicals, such as flavone, apigenin, kaempferide and chalcone, have potent antiestrogenic activity. However, the antiestrogenicity of these compounds does not correlate with their ER binding capacity, suggesting alternative signaling as a mechanism for their antagonistic effects. In this study, we examined the effects of these compounds on the transcription factor activator protein-1 (AP-1). Using AP-1-luciferase stable human endometrial adenocarcinoma Ishikawa and human embryonic kidney (HEK) 293 cells, chalcone, flavone and apigenin all stimulated AP-1 activity. Additionally, we determined the effects of the phytochemicals on transcription factors that are downstream targets of various MAPK pathways. To test this, we used HEK 293 cells stably cointegrated with GAL4 transcriptional activation systems of Elk-1, c-Jun or C/EBP homologous protein (CHOP). Chalcone was the only phytochemical that activated all three transcription factors [Elk-1, 2.7-fold ($P < 0.001$); c-Jun, 2.7-fold ($P = 0.025$); CHOP, 3.0-fold ($P = 0.002$)], whereas apigenin stimulated CHOP (3.9-fold; $P < 0.001$), but inhibited phorbol myristoyl acetate–induced c-Jun activity (71%;$P = 0.006$). This work suggests that phytochemicals affect multiple signaling pathways that converge at the level of transcriptional regulation. The ability of flavonoids to regulate MAPK-responsive pathways in a selective manner indicates a mechanism by which phytochemicals may influence human health and disease. J. Nutr. 132: 1848–1853, 2002.

KEY WORDS: • *flavonoid phytochemicals* • *estrogen receptor* • *mitogen-activated protein kinase* • *activator protein-1*

Phytochemicals are plant compounds that can induce symbiosis with nitrogen-fixing bacteria and can function as deterrents against insects, fungi and herbivores (1–3). Flavonoids (a family of phytochemicals whose members consist of structurally similar flavones, isoflavones, chalcones and coumestans) possess hormonal activity. These compounds enter the human diet primarily in the form of legumes (e.g., soy, lentils or peas), sprouts (e.g., alfalfa or broccoli), licorice root, fruits and vegetables (4–8). At the physiologic level, they have been implicated as endocrine-disrupting agents that can cause infertility, reproductive abnormalities, and tumors in animals fed flavonoid-rich diets (9–14). The estrogenic property of flavonoids is further bolstered by their ability to protect against osteoporosis and cardiovascular disease, two benefits normally attributed to the endogeneous estrogen 17β-estradiol (E_2)[3] (1,15–17).

Despite the adverse health effects of flavonoids, epidemiologic evidence indicates a correlation between populations that consume soy-rich diets and lower incidences of hormone-dependent cancers of the breast and prostate (4,15,18,19). Consistent with this information, soy isoflavones have been shown to prevent carcinogen-induced mammary tumor formation (20,21). This suggests that some phytoestrogens may possess antiestrogenic properties. At the molecular level, flavonoids have been shown to interact with and activate/inhibit

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[3] Abbreviations used: AP-1, activator protein-1; BME, Eagle's basal medium; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; DBD, DNA binding domain; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; E_2, 17β-estradiol; ER, estrogen receptor; ERK, extracellular signal regulated protein kinase; FBS, fetal bovine serum; G418, geneticin; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MEM, minimum essential medium; PKC, protein kinase C; PMA, phorbol myristoyl acetate; UAS, upstream activating sequence.

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the estrogen receptor (ER), leading to an increase or decrease in estrogen-regulated gene transcription and cell proliferation (16,22–24). Therefore, the balance of estrogenic and antiestrogenic activities may represent an important aspect of the human health effects of flavonoids.

The classical paradigm of estrogen function involves E_2 binding to the ER, resulting in receptor activation and subsequent expression of target genes by the E_2/ER complex (25). However, peptide hormones such as epidermal growth factor and insulin-like growth factor-1 can activate the ER in the absence of E_2, pointing to alternative intracellular signaling cascades in ER activation (26–30). Indeed, the mitogen-activated protein kinase (MAPK) pathway is required for full activation of the ER-α by E_2 (31,32).

Flavone, apigenin, kaempferide and chalcone are all compounds that belong to the flavonoid class of phytochemicals and can be found in large quantities in such fruits, vegetables and crop species as apples, onions and tea leaves (33). Apigenin and chalcone in particular can be found in large amounts in chamomile and licorice root, respectively (8,34). We previously demonstrated that certain phytochemicals such as flavone, apigenin, kaempferide and chalcone exhibit strong antiestrogenic activity by demonstrating their ability to inhibit both E_2-mediated gene transcription and MCF-7 cell proliferation (35). More interestingly, the antiestrogenicity of these compounds did not correlate with their ability to bind the estrogen receptor. This suggests the utilization of alternate signaling pathways such as the MAPK pathway by these flavonoids to suppress steroid hormone/receptor action. Given our previous findings plus evidence that suggests regulation of MAPK signaling cascades by various phytochemicals (36–40), we began to elucidate the alternate molecular events initiated by these flavonoids by focusing on components of the MAPK pathway. In particular, we were interested in the endpoints of the pathways, i.e., the various transcription factors that are activated by the three main superfamilies of MAPK, which include the extracellular signal regulated protein kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase 1 and 2 (JNK1/2), and p38 (41,42).

The transcription factors of interest include activator protein-1 (AP-1), Elk-1, c-Jun, and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP). The AP-1 transcriptional complex is composed of DNA binding proteins from the Jun and Fos protooncogene families and is required for stimulation of genes after cytokine and growth factor treatments, as well as for oncogene-mediated transformation (43). AP-1 activation is an indicator of extracellular stimuli and target for multiple signaling cascades; it is therefore an excellent marker to demonstrate whether various environmental compounds can potentiate signal transduction cascades. Expression of AP-1-mediated genes is regulated in two ways: *1*) phosphorylation and activation of individual AP-1 components, primarily Jun; and *2*) expression of the AP-1 components, Jun and Fos. Both Jun and Fos are heavily regulated by the MAPK (44). Elk-1, a member of the E26 transformation specific oncoprotein superfamily, is activated by ERK1/2, JNK1/2 and p38 (42,45,46). Additionally, Elk-1 target sites are located in the promoter region of the c-Fos gene. Hence, activation of Elk-1 will lead to the expression of c-Fos (44). c-Jun is activated potently by JNK1/2 and to a much lesser extent by ERK 1/2 and p38 (46,47). CHOP/Gadd153, a member of the C/EBP family, is activated primarily by p38 (42,48,49). The present study investigated the ability of selective flavonoids to regulate these cell-signaling pathways that are involved in the maintenance of the cell cycle and overall homeostasis of various cells.

MATERIALS AND METHODS

Chemicals and reagents. Apigenin-(4′,5,7-trihydroxyflavone), flavone-(2-phenyl-4H-1-benzopyran-4-one), chalcone and kaempferide-(4-methoxy-3,5,7-trihydroxyflavone) were purchased from INDOFINE Chemical Company (Somerville, NJ). All phytocompounds were prepared in dimethyl sulfoxide (DMSO) and added to the media so that the final concentration of solvent did not exceed 1%. Phorbol myristoyl acetate (PMA) was purchased from Sigma Chemical (St. Louis, MO). PD98059 [MAPK kinase (MEK1/2) inhibitor] was purchased from Calbiochem (San Diego, CA).

The GAL4-DNA binding domain (DBD)-containing plasmids pFA2-c-Jun, pFA2-Elk1, pFA2-CHOP and the 5X-GAL4-upstream activating sequence (UAS) reporter plasmid, pFR-luciferase were all purchased from Stratagene (La Jolla, CA). pGL2-AP-1(PMA)-TA-luciferase was purchased from Clonetech (Palo Alto, CA). pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA). G418 sulfate (geneticin; selects eukaryotic cells stably transfected with neomycin-resistant genes) was purchased from Mediatech, (Herndon, VA).

Cell culture. Human embryonic kidney (HEK) 293 cells were cultured in 150 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM). Ishikawa cells were cultured in 150 cm^2 culture flasks in a 1:1 mix of Ham's F12 and Iscove's modified Dulbecco's medium. Both types of media were supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD), as well as essential and nonessential amino acids, sodium pyruvate, penicillin-streptomycin and porcine insulin (10^{-8} mol/L) (Sigma Chemical). The culture flasks were maintained at 5% CO_2 at 37°C.

Generation of stably-transfected cell lines. T-75 cm^2 flasks of Ishikawa cells or HEK 293 cells were transfected in 7 mL of Opti-minimum essential medium (MEM) media (Gibco-BRL, Gaithersburg, MD) with 10 μg of pAP-1(PMA)-luciferase reporter construct and 1 μg of pcDNA3.1 containing neomycin resistance gene using Lipofectamine Reagent (Gibco-BRL, Gaithersburg, MD). T-75 cm^2 flasks of HEK 293 cells were transfected in 7 mL of Opti-MEM medium with 10 μg of 5X-GAL4-UAS-luciferase and 1 μg of a GAL4-Elk, GAL4-c-Jun, or GAL4-CHOP all containing neomycin resistance gene using Lipofectamine Reagent. After 5 h of transfection, all cells were switched to their normal media for 18–24 h to recover. Cells were then switched to G418-containing selection media until all cells in control nontransfected flasks had disappeared. Cells were grown in G418-containing selection media for an additional 2 wk and then pooled, propagated and tested for PMA-induced luciferase activity.

Luciferase assays. As previously described (50), HEK 293 cells or Ishikawa cells were placed in phenol red–free DMEM supplemented with Eagle's basal medium (BME) and MEM amino acids, sodium pyruvate, and 5% dextran-coated charcoal-treated FBS (5% CS-FBS) for 48 h before plating. The cells were plated in 12-well plates at 5×10^5 cells/well in 5% CS-FBS and allowed to attach overnight. The next day cells were treated with phytocompound, PD98059, phytocompound plus PMA, or PD98059 plus phytocompound or PMA for 18–24 h at 37°C. Concentrations of phytochemicals used were based on doses that gave maximal antiestrogenic effects [(35), unpublished data]. PMA was used as a positive control because we showed previously that 32.4 nmol/L PMA gives significant activation of protein kinase C (PKC), downstream MAPK and AP-1 activation (51). The concentration of PD98059 was described previously (52) and was based on the 50% inhibitory concentration previously published (53).

The treatment-containing media were removed and 200 μL of 1X lysis buffer (Promega, Madison, WI) was added to each well and gently shaken for 1 h at room temperature. The cell debris was then pelleted by centrifugation at 15,000 $\times$ *g* for 1 min. Luciferase activity for 30 μL of cell extracts was determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Statistical analysis. Data were analyzed using one-way ANOVA and post-hoc Tukey's multiple comparisons with SPSS for Windows, version 10 (SPSS, Chicago, IL). The data of each stable cell line were separated into two groups to compare both the fold activation of phytochemicals over negative control (DMSO) and the potentiation/inhibition of PMA-induced activity to explore the effects of phyto-

chemicals on a tumor promoter–stimulated cascade. Data are expressed as means ± SEM. Due to large differences in activation, a natural log transformation was required for luciferase assays to obtain normal distribution. Statistically significant changes were determined at the $P < 0.05$ level.

RESULTS

Phytochemical activation of AP-1 in HEK 293 and Ishikawa cells. We tested the ability of four phytochemicals (**Fig. 1**) to induce AP-1 activity in HEK 293-AP-1-luciferase stable cells (54), an estrogen unresponsive cell line, and found that apigenin by itself (25 μmol/L) induced activity the most, stimulating AP-1 5.2-fold ($P < 0.001$) that of the control (**Table 1**). In addition, apigenin (25 μmol/L) gave a 40-fold enhancement of PMA activity ($P < 0.001$). Kaempferide (25 μmol/L) did not induce AP-1 activity.

We next tested the effects of the flavonoids on AP-1–mediated luciferase expression in estrogen-unresponsive Ishikawa human adenocarcinoma uterine cells, stably propagating an AP-1-luciferase response element to extend our studies to another cell line, to demonstrate that these effects were not exclusive to one cell type. Chalcone (10 μmol/L) was the most effective compound because it gave a 21.7-fold ($P < 0.001$) stimulation and enhanced PMA-induced activation by almost 60-fold ($P < 0.001$) (Table 1). Kaempferide (25 μmol/L) again was the only compound that did not affect AP-1 activity in Ishikawa cells. Additionally, treatment with the MEK 1/2 inhibitor PD98059 reduced PMA-stimulated activity 83.8% as well as that of chalcone (92.7%) and apigenin (26.0%) (data not shown).

Elk1, c-Jun, and CHOP activation by phytochemicals in HEK 293 cells. The transcription factor Elk-1 was stimulated the most by chalcone (10 μmol/L), although only 2.4-fold ($P < 0.001$) that of the vehicle (**Table 2**). Kaempferide (25 μmol/L) was an inhibitor of Elk-1, reducing PMA-stimulated activity 91.0% ($P < 0.001$). Apigenin had the unique characteristic of stimulating Elk-1 by itself while inhibiting PMA-induced activity, suggesting competitive inhibition. Similar to Elk-1, chalcone (10 μmol/L) stimulated c-Jun the most (2.7-fold, $P = 0.025$), whereas kaempferide and apigenin at 25 μmol/L inhibited PMA-induced activity by 78.1% ($P = 0.002$) and 70.6% ($P = 0.006$), respectively. In contrast, CHOP was stimulated the most by apigenin at 25 μmol/L (3.8-fold, $P < 0.001$), whereas kaempferide (25 μmol/L) had no effect on induced activity.

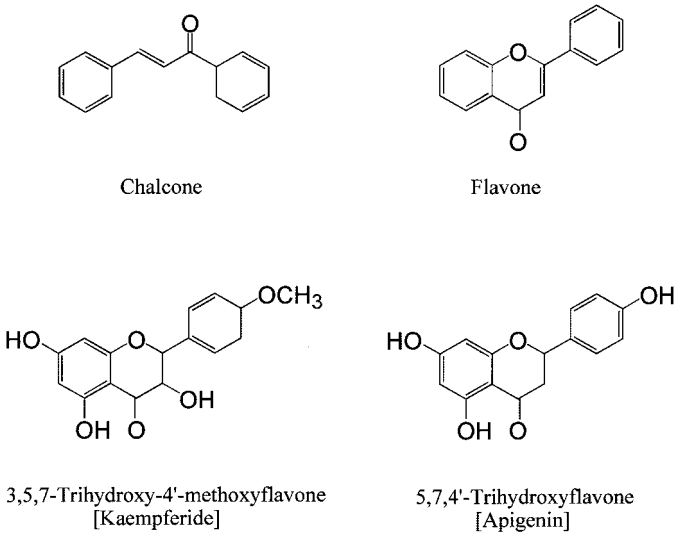

FIGURE 1 Structures of selected flavonoids used in this study.

TABLE 1

Comparison of activator protein-1 (AP-1) activation by phytochemicals with or without phorbol myristoyl acetate (PMA) in human embryonic kidney (HEK) 293 and Ishikawa stable cell lines[1,2]

	HEK 293	Ishikawa
DMSO[3]	1.00 ± 0.0[c]	1.00 ± 0.0[b]
Chalcone	3.83 ± 0.11[b]	21.7 ± 6.03[a]
Flavone	1.92 ± 0.04[c]	1.99 ± 0.29[b]
Kaempferide	1.38 ± 0.2[c]	1.22 ± 0.25[b]
Apigenin	5.24 ± 0.56[a]	1.90 ± 0.31[b]
PMA	33.6 ± 18.3[b]	11.0 ± 2.53[c]
Chalcone + PMA	139 ± 44.2[b]	532 ± 141[a]
Flavone + PMA	115 ± 35.6[b]	45.0 ± 12.2[b]
Kaempferide + PMA	22.0 ± 15.5[b]	9.92 ± 2.0[c]
Apigenin + PMA	579 ± 98.8[a]	108 ± 36.1[b]

[1] Chalcone (10 μmol/L), flavone (25 μmol/L), kaempferide (25 μmol/L) or apigenin (25 μmol/L) were added with or without PMA (32.4 nmol/L). Different superscript letters denote significant mean differences within each cell line at $P < 0.05$.

[2] Values are means ± SEM, $n = 3$, except for Ishikawa: DMSO, PMA and chalcone + PMA treatments ($n = 4$).

[3] DMSO, dimethyl sulfoxide (negative control).

DISCUSSION

Dietary flavonoids have recently gained much attention due to their potential anticancer effects. The molecular mechanisms behind the chemoprotection conferred by these compounds, however, remain largely unknown. Given the role of AP-1 activation in tumor promoter–induced transformation (55), tumor invasion (56,57) and apoptosis (58–60), much research has been targeted toward the effects of dietary flavonoids on AP-1. Studies have shown that flavonoids, such as those found in green and black tea, suppressed activation of AP-1 (61), and that quercetin can inhibit PMA-induced *c-jun* activation and PKC activity (62,63). Our results demonstrated a marked increase in AP-1 activity upon treatment with the flavonoids chalcone, flavone and apigenin in both human endometrial Ishikawa and HEK 293 cells, two estrogen-unresponsive cell lines, indicating the presence of an ER-independent mechanism. In addition, AP-1 activation was further enhanced by cotreatment with PMA. Our results are consistent with the works of Shih et al. (64), in which they demonstrated that quercetin enhanced activation of AP-1–mediated transcription by PMA in HepG2 hepatocarcinoma cells. Several papers demonstrating a critical role for AP-1 in apoptosis raise the possibility that AP-1 activation in this context leads to a cell death or antiestrogenic phenotype rather than a cell proliferation or estrogenic-type effect (58–60,65). Our findings here could thus provide additional explanation of the mechanisms involved in previous work demonstrating the ability of flavonoids such as apigenin to induce apoptosis (38,66). The final interpretation of an environmental signal will ultimately depend in large part on the convergence of unique signals at the level of individual transcription factors such as AP-1.

The ability of select compounds tested here to activate AP-1 suggests the presence of multiple mechanisms in phyto-

TABLE 2

Comparison of transcription factor activation by phytochemicals with or without phorbol myristoyl acetate (PMA) in human embryonic kidney (HEK) 293 stable cell lines[1,2]

	Elk-1	c-Jun	CHOP
DMSO[3]	1.00 ± 0.0[b]	1.00 ± 0.0[b]	1.00 ± 0.0[b]
Chalcone	2.39 ± 0.32[a]	2.68 ± 0.06[a]	2.78 ± 0.4[a]
Flavone	1.66 ± 0.08[a]	2.10 ± 0.54[ab]	2.66 ± 0.26[a]
Kaempferide	1.11 ± 0.14[b]	0.85 ± 0.13[b]	0.86 ± 0.19[b]
Apigenin	2.19 ± 0.2[a]	1.26 ± 0.35[ab]	3.82 ± 0.81[a]
PMA	48.1 ± 9.7[ab]	8.4 ± 1.52[a]	4.04 ± 0.49[ab]
Chalcone + PMA	103 ± 2.18[a]	18.0 ± 0.04[a]	6.42 ± 0.38[a]
Flavone + PMA	61.3 ± 16.7[ab]	8.11 ± 1.46[a]	7.22 ± 0.33[a]
Kaempferide + PMA	3.62 ± 0.28[c]	1.62 ± 0.04[b]	3.57 ± 0.54[b]
Apigenin + PMA	32.9 ± 10.1[b]	2.18 ± 0.06[b]	7.21 ± 1.11[a]

[1] Chalcone (10 μmol/L), flavone (25 μmol/L), kaempferide (25 μmol/L) or apigenin (25 μmol/L) were added with or without PMA (32.4 nmol/L). Different superscript letters denote significant mean differences within each cell line at $P < 0.05$.

[2] Values are means ± SEM, $n = 3$, except for Elk-1: DMSO, flavone, PMA and flavone + PMA treatments ($n = 4$) and C/EBP homologous protein (CHOP): chalcone, kaempferide ($n = 4$) and DMSO, flavone, apigenin and PMA treatments ($n = 5$).

[3] DMSO, dimethyl sulfoxide (negative control).

chemical signaling. To further explore this concept, we utilized the HEK 293 cells stably cointegrated with GAL4-UAS-luciferase reporter plasmids as well as GAL4-DBD-plasmids containing Elk-1, c-Jun, or CHOP. Elk-1, c-Jun and CHOP are major endpoints of the ERK, JNK and p38 MAPK signaling pathways and an activation profile will give insight into the effect each phytochemical has on a cell. Chalcone was the only phytochemical tested that stimulated all of the transcription factors, suggesting diverse activity. Kaempferide had little activity by itself, but like apigenin, it inhibited PMA-induced activity of Elk-1 and c-Jun. This correlates with previous findings that showed apigenin inhibits MAPK signaling and in particular PMA-induced c-Jun activity (37,38,62,67). Not surprisingly, kaempferide, a compound closely related to apigenin, also caused this suppression. Elk-1 is a major target of the MAPK ERK, which is commonly activated in response to proliferative signals (68,69). The ability of kaempferide and apigenin to inhibit the tumor promoter PMA-induced activation of Elk-1 suggests that possibly the antiestrogenic-type phenotype we observed with these compounds was due in part to their ability to inhibit other proliferative signaling pathways such as Raf-MEK-ERK. Interestingly, chalcone, flavone and apigenin all significantly activated CHOP, suggesting a role for p38 in phytochemical signaling; p38 is most often involved in stress-response signaling and its activation can lead to an antiproliferative state similar to the effect caused by antiestrogens (68,69). Hence, the antiestrogenic phytochemicals may function through a shift in proliferative/antiproliferative mechanisms involving MAPK pathways rather than through a classical ER-inhibition mechanism. Both classical and nonclassical mechanisms could then work either separately or together to inhibit hormone-driven proliferation as both our laboratory and others have demonstrated (35,38,70,71). The observation that phytochemicals function through specific MAPK signaling mechanisms is further supported by our studies in which MEK 1/2 inhibitor blocked chalcone- and apigenin-induced AP-1 activity, suggesting the involvement of the Raf-MEK-ERK pathway in AP-1 regulation by these agents. The inhibition of apigenin-induced AP-1 activity may have been less prominent due to a lower initial activation compared with PMA or chalcone.

Interestingly, members of the flavonoid class of phytochemicals have been shown to possess both estrogenic and antiestrogenic properties, indicating that slight changes in chemical structure could lead to a major shift in the response of a cell. Here, we have examined the antiestrogenic properties of two flavones (flavone, apigenin), one flavonol (kaempferide) and one chalcone (chalcone). Of these four compounds, three contain a flavone backbone (flavone, apigenin, kaempferide), whereas the fourth, chalcone, similarly has two phenol groups extending away from the oxygen molecule of a ketone. This is in contrast to the more estrogenic flavonoids, the isoflavones (e.g., genistein, daidzein), which have phenol rings adjacent to the ketone. Structural knowledge of this nature could eventually be used to predict or select for natural designer signal transduction modulators.

The stable cell line system we created and used here allowed us to quickly and efficiently test various chemicals for their ability to affect multiple signaling pathways involved in the control of cell cycle processes, in particular, endpoints of the MAPK cascades. This system eliminated the variability of transient transfections, saving both time and money. Transcription factor activation profiles help us to better understand the type of signal an environmental agent causes and thus the overall effect a compound has on a cell.

In summary, the data presented here indicate alternative signaling mechanisms induced by dietary phytochemicals, more specifically, the flavonoids. Using a novel stable cell line system, various flavonoids were shown to activate the transcription factor AP-1, while having differential effects on transcription factors known to be targets for discrete MAPK cascades. The ability of flavonoids to selectively regulate MAPK-responsive pathways suggests a mechanism by which dietary phytochemicals may influence human health and disease.

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