Arch Pharm Res Vol 29, No 8, 633-644, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Modulation of Activator Protein-1 (AP-1) and MAPK Pathway by Flavonoids in Human Prostate Cancer PC3 Cells

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(Received August 24, 2005)

In last couple of decades the use of natural compounds like flavonoids as chemopreventive agents has gained much attention. Our current study focuses on identifying chemopreventive flavonoids and their mechanism of action on human prostate cancer cells. Human prostate cancer cells (PC3), stably transfected with activator protein 1 (AP-1) luciferase reporter gene were treated with four main classes of flavonoids namely flavonols, flavones, flavonones, and isoflavones. The maximum AP-1 luciferase induction of about 3 fold over control was observed with 20 μM concentrations of quercetin, chrysin and genistein and 50 μM concentration of kaempferol. At higher concentrations, most of the flavonoids demonstrated inhibition of AP-1 activity. The MTS assay for cell viability at 24 h showed that even at a very high concentration (500 μM), cell death was minimal for most of the flavonoids. To determine the role of MAPK pathway in the induction of AP-1 by flavonoids, Western blot of phospho MAPK proteins was performed. Four out of the eight flavonoids namely kaempferol, apigenin, genistein and naringenin were used for the Western Blot analysis. Induction of phospho-JNK and phospho-ERK activity was observed after two hour incubation of PC3-AP1 cells with flavonoids. However no induction of phospho-p38 activity was observed. Furthermore, pretreating the cells with specific inhibitors of JNK reduced the AP-1 luciferase activity that was induced by genistein while pretreatment with MEK inhibitor reduced the AP-1 luciferase activity induced by kaempferol. The pharmacological inhibitors did not affect the AP-1 luciferase activity induced by apigenin and naringenin. These results suggest the possible involvement of JNK pathway in genistein induced AP-1 activity while the ERK pathway seems to play an important role in kaempferol induced AP-1 activity.

Key words: Flavonoids, AP-1, MAPK, JNK, ERK

INTRODUCTION

Activator protein-1 (AP-1) transcription factors comprise a family of ubiquitously expressed proteins that include the Jun (e.g., cJun, JunB, JunD) and Fos (e.g., cFos, FosB, Fra-1, Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K) and ATF (ATF2, LRF1/ATF3) proto-oncoproteins (Karin *et al.*, 1997). The resulting homodimeric (Jun-Jun) or heterodimeric (Jun-Fra or Fos-Jun) complex binds a palindromic DNA sequence, known as 12-*O*-tetredecanoylphorbol-13-acetate (TPA) responsive element (Foletta, 1996). This element is known to be present within the regulatory region of several genes including c-jun. The AP-1 complex mediates responses to cellular signals by binding to DNA and producing changes in gene transcription that ultimately lead to physiological functions in the cell. Several lines of evidence indicate that AP-1 activity is involved in many different cellular processes including cell proliferation, differentiation, apoptosis, and stress responses (Wisdom 1999; Shaulian and Karin, 2001, 2002). Depending upon the composition of the AP-1 complex, different genes could be activated as a result of AP-1 binding (Kaminska *et al.*, 2000). Although the relevance of AP-1 in human diseases is not completely understood, it is thought that AP-1 proteins may play a significant role in the pathogenesis of human cancers. Dong and co-workers demonstrated that blocking AP-1 activity by pharmacological or biological inhibitors impaired neoplastic trans-

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formation by tumor promoters such as UV light and PMA (Dong *et al.*, 1995). On the other hand a report by Zerbini *et al.* showed that the aberrant activation of AP-1 transcription factors in human prostate cancer cells results in deregulation of interleukin-6 (IL-6), which is associated with androgen-independent human prostate cancer (Zerbini *et al.* 2003). This suggests that regulation of AP-1 activity may be important in prostate cancer development/progression.

It is well established that androgen is the major growth and survival factor for the normal prostate and that the prostate does not develop and will atropy without androgen support (Ross *et al.*, 1998). In the normal prostate androgen action is a paracrine process and it has varying effects on different cell types. In stromal cells the binding of the androgen receptor by its cognate ligand initiates signaling pathways that regulate epithelial cell survival and proliferation (Litvinov *et al.*, 2003). In the nuclei of the secretory cells, the androgen receptor is involved in the transcriptional regulation of prostate specific markers such as PSA and human glandular kalikrein-2. However, in the nucleus of the secretory cells, androgen receptor suppresses cell proliferation (Ross *et al.*, 1998). During prostatic carcinogenesis, androgen receptor binding in the nuclei of secretory cells no longer inhibits cell proliferation (Gonzalgo and Isaacs, 2003; Nelson *et al.*, 2003; Isaacs and Isaacs, 2004; Uzgare and Isaacs, 2004). Thus, the normal paracrine function of the androgen receptor signaling is converted into an autocrine function whereby the binding of androgen receptor within the cancer cells directly stimulates their survival and proliferation (Gao *et al.*, 2001). Thus, androgen withdrawal produces an initial response of apoptosis in these androgen-dependent cells. Unfortunately, this androgen-dependent cell survival is nearly universally followed by a relapse to a refractory state in which the cancer cells continue to survive and proliferate despite a low, circulating androgen environment. This stage though is not commonly associated with reduced androgen receptor expression (Isaacs and Isaacs, 2004). Several lines of evidence suggest multiple molecular changes that result in these cancer cells acquiring alternative ways of activating survival and proliferative pathways without requiring physiological levels of circulating androgens (Gonzalgo and Isaacs, 2003). Thus, identifying the signal transduction pathways responsible for the survival and proliferation of androgen-independent prostate cancer cells is critical for future targeted drug development.

Race, age and diet are identifiable risk factors associated with prostate cancer occurrence (Agarwal 2000). An increasing body of evidence suggests that several essential nutrients present ubiquitously in fruits, vegetables and other beverages such as tea and wine can suppress prostate cancer development (Greco and Kulawiak, 1994). Identifying these factors and understanding their chemopreventive mechanisms presents a noninvasive strategy for decreasing the incidence and severity of this disease.

Strong evidence from experimental and epidemiological studies have established a positive link between consumption of several yellow-green vegetables that are rich in flavonoids and fresh fruits especially grapes and the reduced incidence of cancer. Procyanidins, a diverse group of flavonoids, are the principal constituents of grape seed extract and are also prevalent in a number of fruits and vegetables (Agarwal, 2000; Souquet *et al.*, 2000; Joshi *et al.*, 2001). A study performed by Agarwal and coworkers demonstrated that grape seed extract induced apoptotic death in DU145 human prostate cancer cells (Agarwal *et al.*, 2002). A more recent study by Rana *et al.* demonstrated that grape seed extract inhibited advanced human prostate tumor growth and angiogenesis and also inhibited insulin-like growth factor binding protein-3, high levels of which is known to be positively correlated with increased risk of prostate cancer (Singh *et al.*, 2004). Another study performed by Huang *et al.* demonstrated that baicalin (baicalein 7-*D*-β-glucuronate) could inhibit the proliferation of several human prostate cancer cells including PC3, DU145, LNCaP (Chan *et al.*, 2000; Chen *et al.*, 2001).

Taken together, there is an imminent need for chemopreventive strategies for prostate cancer and it is almost certain that dietary constituents play a significant role in trying to fulfill this need. In line with this, we screened several flavonoids for their potential chemopreventive properties on human prostate cancer cells. Thus, the goal of this study was to investigate the chemopreventive mechanims and the signaling pathways induced by these chemopreventive agents within the cell. In order to achieve the specific aims of this study, we stably transfected androgen-independent human prostate cancer cell line (PC3) with AP-1 luciferase reporter gene. This cell line was then used to screen eight flavonoids belonging to four different classes namely flavonols, flavones, flavonones and isoflavones. Since AP-1 activity can be induced by several growth factors, cytokines, bacterial and viral infection and a variety of physical and chemical stresses and since these stimuli can activate MAPK cascades that enhance AP-1 activity through phosphorylation of a variety of substrates, we studied the modulation of the MAPK pathway by these flavonoids as well.

MATERIALS AND METHODS

Materials

Quercetin, kaempferol, apigenin, chyrsin, naringenin, hesperitin, genistein, biochanin, SP600125 (specific JNK inhibitor) and U0126 (MEK inhibitor) were purchased from

Sigma chemicals Co. (St. Louis, MO). All other chemicals were purchased from commercial sources and were of analytical grade. Human prostate cancer cell line PC3 was obtained from American Type Culture Collections (Manassas, VA). AP-1 luciferase reporter plasmid construct containing AP-1 consensus binding site was a kind gift provided by Dr. Anning Lin (University of Chicago, Chicago, IL).

Cell culture and stable transfection

PC3 cell line was maintained in Minimal Essential Medium supplemented with 10% bovine serum albumin and antibiotics at 37°C with 5% CO_2. AP-1 luciferase construct containing the AP-1 consensus binding site (-TGACTCA-) and pcDNA3.1 neomycin plasmid were co-transfected into PC3 cells by Lipofectamine TM 2000 (LF2000, Invitrogen life technology, Carlsbad, CA) following the manufacturer's instruction and stable clones were selected with 0.5 mg/mL of G418 sulfate (Invitrogen Life Technology, Carlsbad, CA). One of the stable clones was subcultured and used for further studies.

AP-1 Luciferase assay

The cells were subcultured in 6-well plates at a density of 1×10^5 cells/well. 12-16 h prior to treatment, the medium was changed to a starving medium containing 0.5% FBS. The cells treated with the various flavonoids and incubated for 24 h else they were pre-treated with the JNK or MEK inhibitors for 30 minutes and then treated with the respective flavonoids and then incubated for 24 h. Luciferase activity was assayed with a luciferase kit from Promega (Madison, WI) by using a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase acivity was normalized against protein concentration and expressed as fold induction over control cells.

MTS Assay for cell viability

PC3 AP1 cells were seeded in 24 well plates at a density of 10^5 cells/well. 12-16 h prior to treatment, the medium was changed to a starving medium containing 0.5% FBS. The cells were incubated with different doses of the flavonoids for a period of 24 h. The MTS [3, 4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed with Cell Titer 96 Aqueous nonradioactive cell proliferation assay kit (Promega Crp., Madison, WI) by manufacturer's instruction. After 2 h, the absorbance was measured at 490 nm with m Quant ELISA reader (BIO-TEK Instruments, Inc., Madison, WI). The cell viability was determined by the percentage of treated over the control that was treated with DMSO (0.1%).

Western blot analysis

The cells were then washed with ice-cold PBS, after treatment and harvested with 200 μL of a lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonylfluoride and 0.5% Triton-X 100. The supernatant was collected and twenty micrograms of total protein, as determined by Bio-Rad protein assay, was mixed with 4x loading buffer, and pre-heated at 95°C for 3 min. The samples were then loaded on a 10% SDS–polyacrylamide gel, and run at 200 V. The proteins were transferred onto PVDF membrane for 1.5 h using semi-dry transfer system (Fisher, Pittsburg, PA). The membrane was blocked in 5% bovine serum albumin solution for 1 h at room temperature, and then incubated overnight at 4°C with indicated primary antibody (1:1000 dilution). After hybridization with primary antibody, the membrane was washed with TBST (Tris buffered-saline Tween-20) three times, and incubated with HRP-labeled secondary antibody for 45 min at room temperature. Final detection was performed with ECLTM (Enhanced Chemiluminescence) Western blotting reagents (Amersham Pharmacia Biotech).

Statistical analysis

For statistical analysis of the data, ANOVA followed by Bonferroni's post-hoc test was used at a significance level of $P<0.05$. The data represent mean ± SE.

RESULTS

Effects of chemopreventive agents on transcriptional activation of AP-1 in PC3 cells

To investigate and compare the effects of various natural chemopreventive agents on the transcriptional activation of AP-1, we constructed a PC3 cell line that was stably transfected with the AP-1 luciferase reporter gene. TPA (12-O-tetradecanoylphorbol-13-acetate), LPS (lipopolysacchrides) and H_2O_2 (hydrogen peroxide) are known to activate AP-1 transcriptional activation in several cell models. However, according to a study performed in our laboratory, none of these agents increased the transcriptional activity of AP-1 dramatically in the PC3 cell line (unpublished results). Hence these agents could not be used as positive controls in any of our experiments.

Four different groups of flavonoids namely flavonols, flavones, flavanones and isoflavones were tested for their effects on AP-1 luciferase activity. Fig. 1 shows the structures of all the flavonoids assessed for their chemopreventive properties in this study. Fig. 2a demonstrates the effects of flavonols on AP-1 luciferase activity. Quercetin and Kaempferol are both flavonols. Both compounds have similar C rings but differ in their B ring structure in that quercetin is a 3', 4' diol while kaempferol has one OH

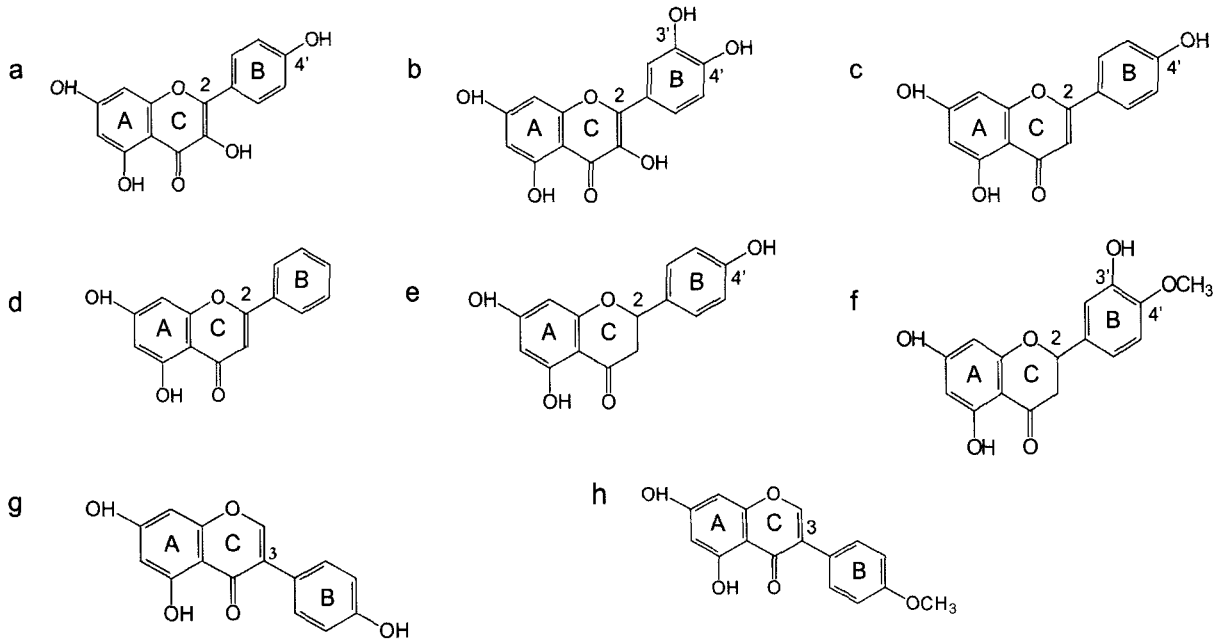

Fig. 1. Structures of the various chemopreventive compounds used in this study (a) quercetin, (b) kaempferol, (c) chrysin (d) apigenin, (e) naringenin, (f) hesperitin, (g) genistein, (h) biochanin

group at position 4'. Quercetin and Kaempferol both induced maximal AP-1 luciferase activity ~3 fold at 20 μM and 50 μM concentrations respectively. At higher concentrations, both compounds caused a slight inhibition of AP-1 luciferase activity.

Fig. 2b demonstrates the effects of flavones on AP-1 luciferase activity. Apigenin and Chrysin are both flavones. They differ from quercetin and kaempferol in that they lack the hydroxyl group on ring C. Apigenin however has a 3' hydroxyl group on ring B which chrysin lacks. Chrysin induced AP-1 activity ~3 fold over control at 20 μM concentration.

Fig. 2c demonstrates the effects of isoflavones on AP-1 luciferase activity. Isoflavones differ from flavones in that the phenyl ring is attached to position 3 on ring C instead of position 2. The hydroxyl group on ring B is methylated in biochanin. Genistein induced AP-1 activity to almost equal levels (~3 fold) at both 20 μM and 50 μM concentrations. Biochanin on the other hand did not induce any AP-1 activity.

Fig. 2d demonstrates the effects of flavonones on AP-1 luciferase activity. Naringenin and Hesperitin are both flavonones. The structural difference between flavonones and flavones is that in flavonones the double bond in ring C is reduced. Hesperitin differs from naringenin in that the hydroxyl group at position 3' on ring B is methylated. Neither naringenin nor hesperitin demonstrated a pronounced effect on the transcriptional activation of AP-1.

Cell viability assay

To investigate whether the slight inhibition of AP-1 luciferase activity modulated by the flavonoids at higher concentrations is due to cytotoxicity, we measured the cell-viability of PC3-AP1 cells using the MTS assay. The results are as depicted in Fig. 3. The data is expressed as percent cell viability as compared to the control cells which were treated with 0.1% DMSO. The concentrations of the flavonoids ranged from 10 μM to 500 μM. Treatment with 500 μM chrysin resulted in ~70% cell viability as opposed to kaempferol and apigenin that resulted in ~78% and ~82% cell viability. All the other flavonoids even at the highest concentrations produced negligible cell death. Interestingly at 100 μM concentration, the highest concentration used for luciferase assay, chrysin and kaempferol demonstrated ~80% cell viability while all the other flavonoids exhibited ~90% or >90% cell viability.

Activation of JNK

To investigate whether the increased AP-1 activity by these flavonoids can be correlated to JNK activity, we examined the phosphorylation of JNK1/2 using western blot analysis of PC3-AP-1 cells. We tested and compared the effects of four of the eight flavonoids namely kaempferol, apigenin, genistein and naringenin. Apigenin was chosen over chrysin, although chrysin clearly demonstrated better AP-1 transcriptional activation only in order to include atleast one flavonoid that did not follow the general pattern of AP-1 activation among its class. Their effects on the phosphorylation of JNK1/2 and the results are as depicted in Fig. 4a. In the control cells, only small amounts of both 54 and 46 kDa isoforms of phospho-JNK1/2 were detected. However both these isoforms of phospho-

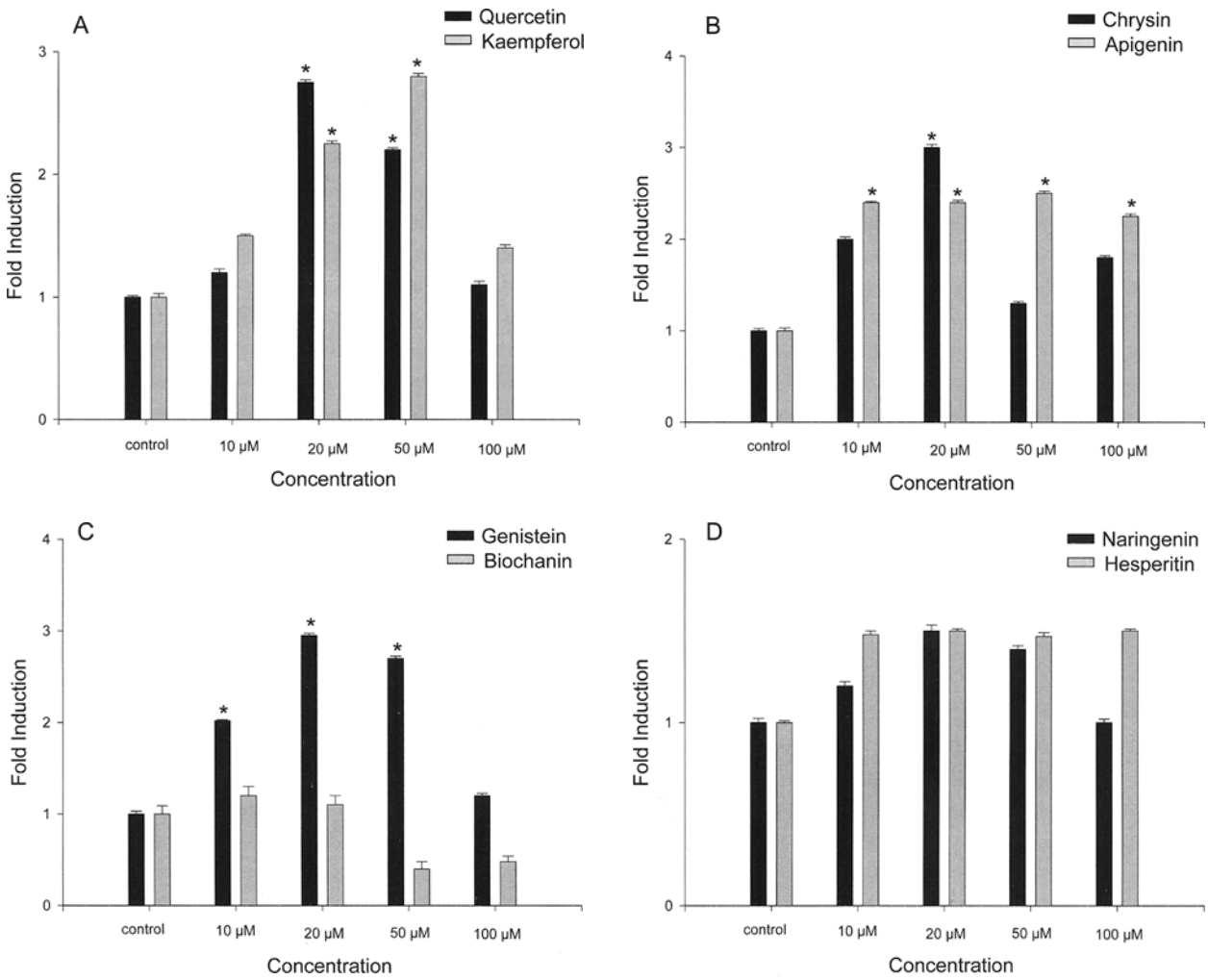

Fig. 2. **(A)** Effects of flavonols quercetin and kaempferol; **(B)** Effects of flavones chrysin and apigenin; and **(C)** Effects of isoflavones genistein and biochanin, on the AP-1 transcriptional activity in PC3 cells stably transfected with AP-1-luciferase reporter gene. Cells were serum starved for 12-16 h with 0.5% serum-MEM medium and then treated with each compound and incubated for 24 h. Values are representative results performed in triplicates with very similar results from five separate independent experiments. * Significantly different compared to control ($p<0.05$) by Bonferoni's post hoc test.

JNK were induced by all the four flavonoid treatments. Kaempferol dose-dependently induced the 54 and 46 kDa isoforms of phospho-JNK1/2. Maximum activation of phospho-JNK was observed at 20 μM concentration of kaempferol. Naringenin and apigenin both showed slight activation of phospho-JNK however they lacked dose-dependency. At higher concentrations, the phosphorylation of the JNK was found to be weak. Genistein also induced maximum phospho-JNK at 10 μM concentration. At higher than 10 μM concentration, there was very weak phosphorylation of JNK. The effects of these flavonoids on the protein level of JNK in PC3 AP1 cells are as shown in Fig. 4c.

Activation of ERK

In order to test whether the modulation of AP-1 activity by these compounds involves phosphorylation of ERK protein, we performed similar Western blot analysis of the PC3-AP1 cells treated with varying concentrations of the four flavonoids. The results are as depicted in Fig. 4b. In the control cells treated with solvent DMSO only small amounts of 44 and 42 kDa isoforms of phosphorylated ERK protein was detected. Most of the flavonoids seem to activate phospho-ERK though no significant dose-dependency was observed. Slight activation of phospho-ERK was observed with 20 μM Kaempferol and 50 μM Apigenin. At higher concentrations there was decreased phosphorylation of ERK. The luciferase activity results demonstrate that Kaempferol induced maximum AP-1 transcriptional activity at 50 μM though kaempferol induced AP-1 activity at 20 μM was also found to be statistically significant. Genistein and Naringenin demonstrated slight activation of phospho-ERK at 10 μM and 20 μM, respectively. Again, at higher concentrations there was weak

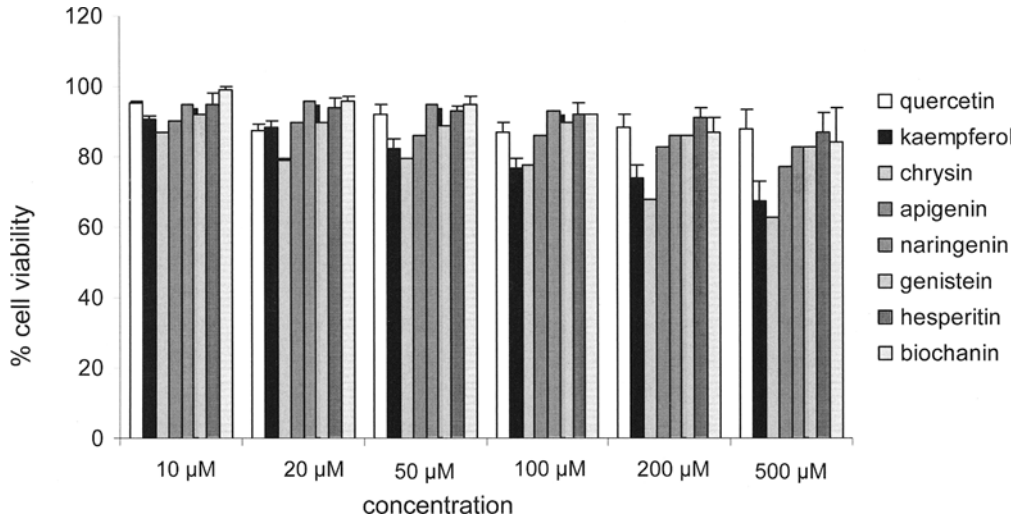

Fig. 3. Effect of various chemopreventive agents on cell viability of PC3 cells stably transfected with AP-1-luciferase reporter gene. Cells were serum-starved for 12-16 h with 0.5% serum-MEM medium and then treated with different concentrations of the flavonoids for 24 h after which the cells were washed thoroughly with ice-cold PBS and incubated with MTS reagent for 2 h and the absorbance was measured at 490 nm.

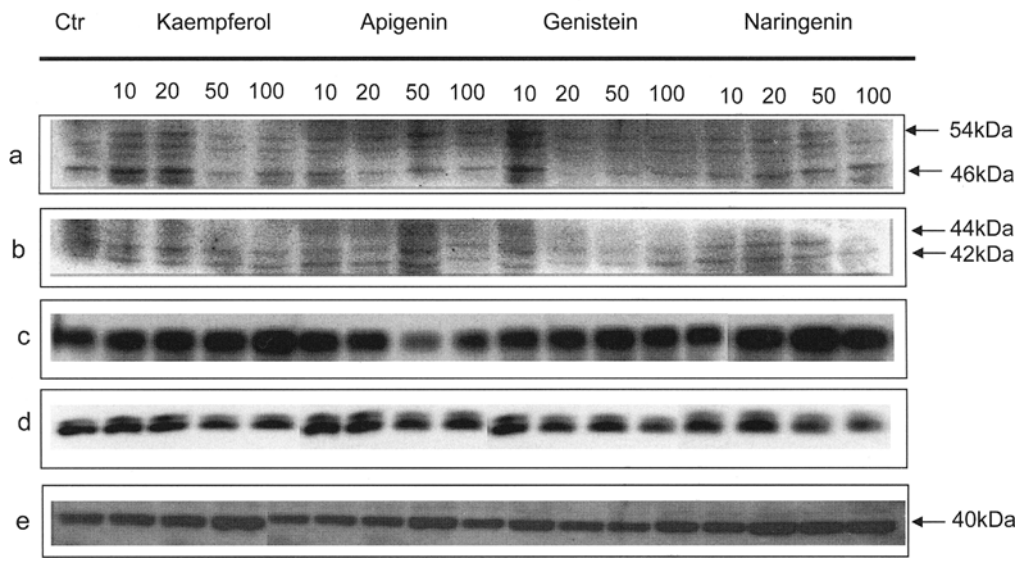

Fig. 4. Effect of the various chemopreventive agents on the MAPK activity (a) Western blot of phospho-JNK, (b) Western blot of phospho-ERK (c) Western blot of total JNK, (d) Western blot of total ERK and (e) Western blot of β-actin. Briefly, after 12-16 h of serum-starvation, the cells were treated with varying concentrations of the flavonoids for 2 h. Cell extracts were then subjected to Western blot analysis. Control cells were treated with vehicle 0.1% DMSO. Actin was used to ensure equal protein loading.

phosphorylation of ERK. Fig. 4d demonstrates the effects of these flavonoids on the protein levels of ERK. None of these flavonoids induced phosphorylation of p38, another member of the MAPK family (results not shown).

Effects of flavonoids on phosphorylated MEK

In order to confirm the involvement of ERK pathway in the AP-1 transcriptional activity induced by these flavonoids, we examined the phosphorylation of MEK. The results are as depicted in Fig. 5. Treatment with kaempferol resulted in activated MEK. Interestingly, 50 μM kaempferol demonstrated a slight reduction in activated MEK. Ongoing studies in our laboratory are focused towards understanding such biphasic nature of MEK activation followed by kaempferol treatment Apigenin and genistein showed an induction of phospho-MEK at 50 μM and 10 μM, respectively. To further understand the involvement of MAPK cascades following treatment by flavonoids, specific inhibitors to the same were used.

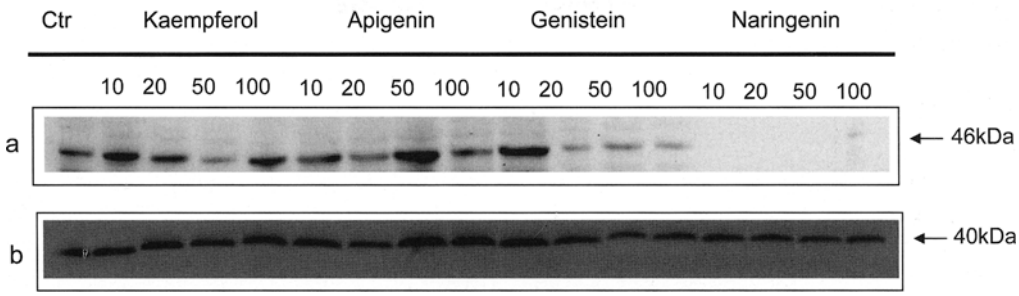

Fig. 5. Effect of various chemopreventive agents on MEK activity using Western blot. Briefly, after 12-16 h of serum starvation the cells were treated with varying concentrations of flavonoids for 2 h. Cell extracts were then subjected to Western blot analysis to assess levels of phosphorylated MEK. Control cells were treated with vehicle 0.1% DMSO. Actin was used to ensure equal protein loading.

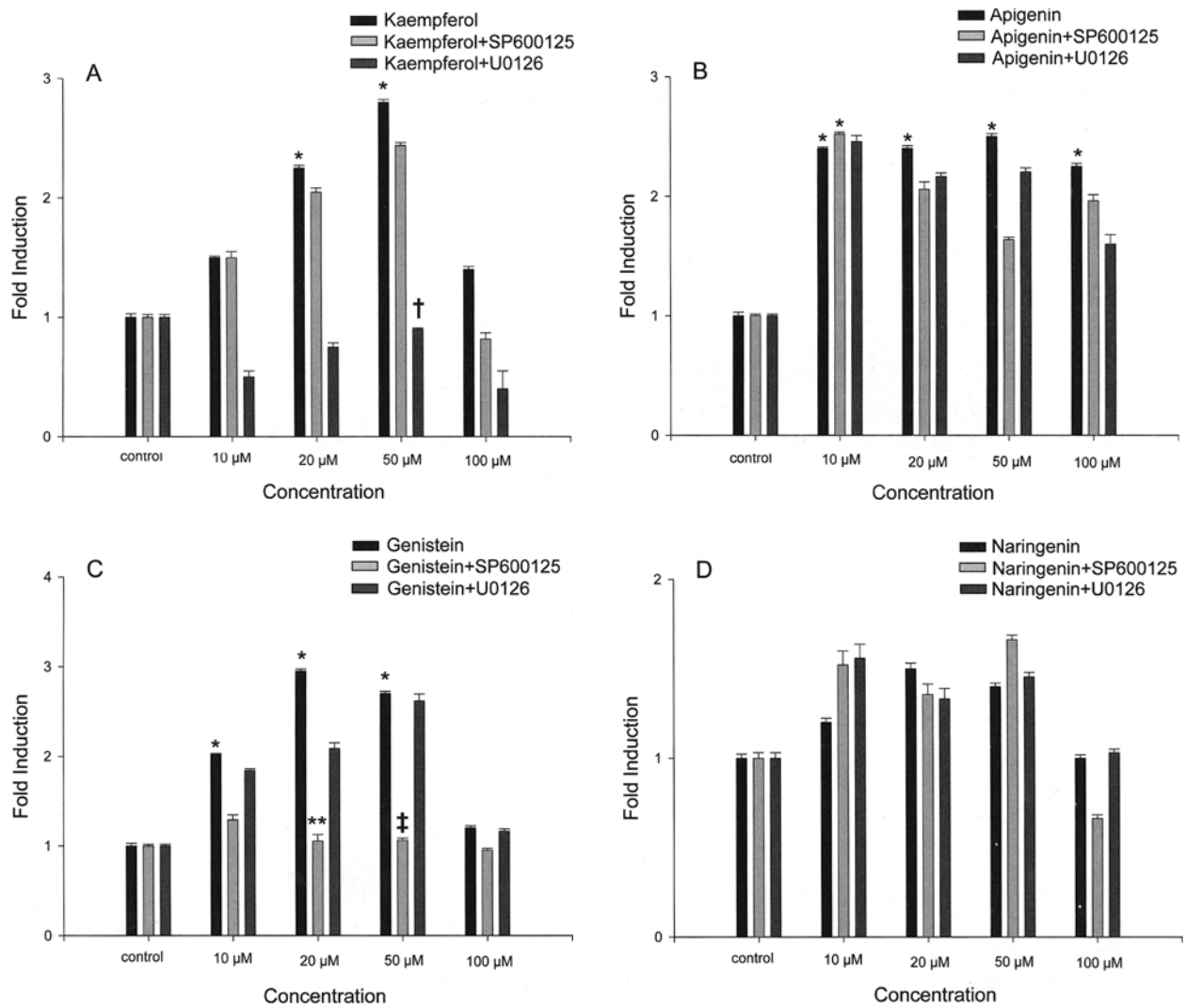

Fig. 6. Effect of specific JNK and MEK inhibitor on AP-1 activity induced by **(A)** kaempferol; **(B)** apigenin; **(C)** genistein; and **(D)** naringenin. PC3 cells stably transfected with AP-1 were pre-treated with SP600125 (JNK inhibitor) or U0126 (MEK inhibitor) for 30 minutes followed by the flavonoid and incubated for 24 h. Values are representative results performed in triplicates with very similar results from five separate independent experiments. *Significantly different compared to control; † Significantly different compared to 50 μM kaempferol ($p < 0.05$); ** Significantly different compared to 20 μM ($p < 0.05$) and ‡ Significantly different compared to 50 μM genistein ($p < 0.05$) by Bonferoni's post hoc test.

Effects of specific JNK and MEK inhibitors on the AP-1 transcriptional activity induced by flavonoids

In order to corroborate the results from the Western blot analysis and the luciferase activity and to discern whether or not the MAPK pathway plays an important role in the transcriptional activation of AP-1 by the flavonoids, we treated the cells with specific JNK and MEK inhibitors and observed their effects on the AP-1 luciferase activity. The cells were pretreated with 10 μM SP600125 (specific JNK inhibitor) or 10 μM U0126 (specific MEK inhibitor) and then incubated with the respective flavonoids for a period of 24 h. The results are as depicted in Fig. 6. Fig. 6a demonstrates the effects of these inhibitors on kaempferol induced AP-1 transcriptional activity. Treatment with the MEK inhibitor markedly abolished kaempferol induced luciferase activity while the JNK inhibitor did not show any pronounced effect. Hence it may be deduced that kaempferol induced AP-1 activity predominantly by the MEK-MAPK pathway. Interestingly, neither the JNK inhibitor nor the MEK inhibitor reduced apigenin induced AP-1 transcriptional activity. In the case of genistein, treatment with the JNK inhibitor effectively abolished AP-1 activity even at 10 μM concentration. The MEK inhibitor did not show much effect on genistein induced AP-1 activity. Hence though genistein did slightly induce MEK, this pathway may not result in the activation of AP-1. Thus, the JNK-MAPK pathway may play a predominant role in activating AP-1 upon treatment with genistein. Treatment with neither JNK nor MEK inhibitor effectively abolished AP-1 activity induced by Naringenin. The luciferase assays results for naringenin too demonstrate a lack of dose dependency. Thus by treatment with these pharmacological inhibitors, it may be deduced that though apigenin and naringenin can activate the JNK and ERK-MAPK pathways, these pathways may not play a pivotal role in the transcriptional activation of AP-1 by these agents.

DISCUSSION

Flavonoids are a class of natural compounds that occur ubiquitously in food, plants and vegetables. Chemically, they have a phenylchromanone structure (C_6-C_3-C_6) and usually have at least one hydroxyl group substituent or a hydroxyl group derivative such as a methoxy group (Nikolic and van Breemen, 2004). They have been found to possess various clinically relevant properties such as anti-tumor, anti-platelet, anti-ischemic, and anti-inflammatory activities and these effects are believed to be an outcome of their antioxidant properties (Kubo *et al.*, 1985; Motoo and Sawabu, 1994; Shi *et al.*, 1995; Gabrielska *et al.*, 1997). Individual differences in the effects of flavonoids result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and the extent of alkylation and/or glycosylation of these groups. Compelling data from *in vitro* and *in vivo* laboratory studies and epidemiological investigations indicate that flavonoids exert important effects on cancer chemoprevention and therapy.

Silibinin, a component of silymarin, is a classic flavonoid exhibiting potential anticancer efficacy against prostate cancer and various other cancers too. It is also sold as a dietary supplement in the United States and Europe (Agarwal, 2000). Both silymarin and silibinin are exceptionally well tolerated and are reported to be non-toxic in acute, chronic, sub-chronic tests in various animals with no known LD_{50} (Agarwal *et al.* 1994). It has been shown that silibinin/silymarin feeding to mice upto 2 gm/kg did not show any signs of toxicity and also that it is physiologically available in different organs of the mice (Hahn *et al.*, 1968; Agarwal *et al.*, 2003). Likewise, several epidemiological studies suggest that consumption of green tea lowers the risk of cancer (Liao *et al.*, 1995; Katiyar *et al.*, 1997; Conney *et al.*, 1999; Saleem *et al.*, 2003). Soy isoflavones including genistein, daidezin and glycitein, mainly derived from soybean have been found to inhibit cancer growth *in vivo* and *in vitro* (Messina *et al.*, 1994; Onozawa *et al.*, 1998). Geller and co workers reported that genistein inhibits the growth of malignant and benign prostatic hyperplasia (Geller *et al.*, 1998). Consumption of olives/ and or olive oil containing large amounts of flavone apigenin is also associated with decreased risk of cancer.

It is increasing becoming clear that these dietary agents exert their pleiotropic effects on cancer cells, affecting cell survival and physiological behaviors. However the precise molecular mechanisms of actions of these compounds have not been elucidated, although the data from published literature indicate that these compounds regulate cellular signal transduction pathways such as NF-κB, MAPK, Akt etc (Yu *et al.,* 1996; Kong *et al.,* 2000; Jeong *et al.*, 2004a, 2004b). In this study we demonstrate the effects of various flavonoids on the transcriptional activation of AP-1 and their modulation of the MAPK pathway.

Ishikawa and Kitamura reported that in mesangial cells, quercetin abolished H_2O_2 induced AP-1 activity (Ishikawa and Kitamura, 2000). Here we report that at lower concentrations, quercetin dose-dependently increased AP-1 activity while at higher concentrations slight inhibition was observed. Similarly, kaempferol also induced AP-1 activity at lower concentrations and exhibited some inhibition at higher concentrations. Apigenin increased the transcriptional activity of AP-1 ~2.5 fold over control at 20 μM concentration while chrysin increased the transcriptional activity of AP-1 ~3 fold over control at the same concentration. Similarly, both apigenin and chrysin showed some inhibition of AP-1 activity at higher concentrations. Frigo

and coworkers demonstrated that in endometrial Ishikawa and HEK 293 cells, apigenin markedly increased AP-1 activity and it was further enhanced by cotreatment with phorbol-12-myristate-13-acetate (PMA) (Frigo *et al.*, 2002). The isoflavones - genistein and biochanin and flavononoes - naringenin and hesperitin induced AP-1 activity at lower doses while at higher concentrations all three flavonoids except hesperitin slightly inhibited AP-1 activity. Taken together, most of the flavonoids tested in this report increased AP-1 activity at low doses. This observation is similar to a previous study performed in our laboratory where we demonstrated that in HT-29 colon cancer cells stably transfected with AP-1, EGCG a flavan-3-ol induced AP-1 luciferase activity about 12 fold over control at 100 μM concentration. Co-treating HT-29 cells with EGCG (100 μM) and TPA produced 14 fold increase in AP-1 activity. Likewise sulforafane an isothiocyanate compound also increased AP-1 activity ~3 fold over control and cotreating it with TPA increased AP-1 activity ~10 fold over control (Jeong *et al.*, 2004).

Since all the flavonoids tested in our current study demonstrated some inhibition of AP-1 activity at higher concentrations of 100 μM, we performed the MTS assay to test whether any of this inhibition was due to cytotoxicity. The cells were incubated with high concentrations of flavonoids up to 500 μM for 24 h in keeping with the incubation period used for the luciferase assay. Even at very high concentrations of 500 μM, the flavonoids were not cytotoxic to the cells.

AP-1 activation is often considered as an indicator of external stimuli and several signal transduction pathways converge at the level of this transcription factor. It is, therefore, a potential target for multiple signaling cascades. Thus, it has been shown to be an excellent marker to demonstrate whether various chemopreventive compounds can modulate signal transduction pathways. The ability of the lower doses of flavonoids to activate AP-1 transcriptional activity and higher doses to inhibit AP-1 transcriptional activity without causing cell death, suggests the presence of multiple signaling mechanisms.

Expression of AP-1 mediated genes are regulated in two ways: a) phosphorylation and activation of individual AP-1 components and b) expression of AP-1 components jun and fos. Both jun and fos are heavily regulated by MAPK pathways (Angel and Karin, 1991; Wisdom, 1999). MAPKs are important in controlling the cellular events such as proliferation, differentiation, and apoptosis (Seger and Krebs 1995). ERK activation usually leads to elevated AP-1 activity via c-fos induction. This results in increased synthesis of c-fos, which upon translocation to the nucleus dimerizes with the pre-existing Jun proteins to form AP-1 dimers (Karin and Smeal, 1992; Karin, 1995). ERK1 and ERK2 are widely expressed and are involved in the regulation of meiosis, mitosis and post-mitotic functions in differentiated cells. Several different kinds of stimuli, including growth factors, cytokines and carcinogens activate the ERK1 and 2 pathways. The JNKs were characterized as stress-activated protein kinases on the basis of their activation in response to stress activators as well as inhibition of protein synthesis (Vogt and Bos, 1990; Vogt, 1995; Karin, 1996). JNK is known to bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity.

Nguyen and coworkers demonstrated that in human A549 lung cancer cells, kaempferol induced growth inhibition and apoptosis and this was mediated by the activation by MEK-MAPK pathway (Nguyen *et al.*, 2003). Similarly, here we showed that kaempferol induced AP-1 activity possibly by activation of the MEK-MAPK pathway. Paweletz *et al.* reported by protein microarray analysis that prostate cancer progression was associated with increased AkT and decreased ERK phosphorylation (Paweletz *et al.*, 2001). To further solidify this relationship, Ghosh reported that in poorly differentiated prostate cancer the serine/threonine kinase Akt is highly phosphorylated as compared to ERK (Ghosh *et al.*, 2005). Thus it is possible that in human prostate cancer cells that express low levels of ERK, the chemopreventive agent kaempferol activates the MEK-MAPK pathway leading to the phosphorylation of certain transcription factors that translocate from the cytosol to the nucleus and ultimately activate the transcription of genes that could result in apoptosis of the cells.

Singletary and coworkers demonstrated that in MCF-10F cells, genistein did not activate JNK (Frey and Singletary, 2003), while Croisy and coworkers showed that in A431 cells, genistein and its analogue MD833 activated the JNK pathway (Croisy-Delcey *et al.*, 1997). Our present results show that genistein induced AP-1 activity and the JNK MAPK pathway. Differences in cell types might in part explain the activation of JNK in PC3 and A431 cells as compared to lack of JNK activation in MCF-10F cells.

Boldt and coworkers demonstrated that taxol, an agent that targets microtubules, etoposide, an agent that targets topoisomerase II, and ceramide, a synthetic lipid that is a second messenger to TNF, all induce MAPK signaling cascades that results in an apoptotic response in different cancer cells (Boldt *et al.*, 2002). A more recent report by Mallikarjuna and coworkers established that silibinin treatments in UVB induced tumorigenesis resulted in strong phosphorylation of ERK1/2, JNK and p38. This suggests the possible involvement of the induced MAPKs in apoptotic effects of silibinin (Mallikarjuna *et al.*, 2004). Consistent with these reports, the activation of the MAPK cascade is suggested as a possible mechanism by which

these flavonoids compounds exert their chemopreventive action.

In summary, the PC3 cells stably transfected with AP-1 luciferase reporter gene can be used as a potential tool to screen a variety of chemopreventive agents. It is known that most of these agents modulate intracellular signal transduction pathways potentially leading to chemoprevention. However our study clearly demonstrates that the activation of these pathways could be dependent on several parameters such as the chemical structures of the compounds, concentration and the incubation period. Also since almost every pathway has cross-talk with other signal transduction pathways, the activation or inhibition of one pathway may not completely account for the chemopreventive action of these natural compounds. In the current study we have demonstrated that while kaempferol and genistein may activate the MEK-ERK and JNK MAPK cascades, apigenin and naringenin did not appear activate either pathways. Whether or not other signal transduction pathways are involved and if they are involved, which pathway is more significant towards eliciting the chemopreventive action of these compounds needs further studying. Hence from our current study we infer that each flavonoid compound exhibited a different level of potency in modulating intracellular signaling cascades. The possibility that combination of these compounds may be more effective in slowing prostate cancer progression than single agents cannot be ruled out. More studies in this direction are currently ongoing in our laboratory.

ACKNOWLEDGEMENTS

We thank Dr. Michael Karin (University of California, San Diego) and Dr. Anning Lin (University of Chicago) for providing AP-1 reporter gene construct.

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