Journal of Cellular Biochemistry Supplements 28/29:39–48 (1997)

Suppression of Protein Kinase C and Nuclear Oncogene Expression as Possible Molecular Mechanisms of Cancer Chemoprevention by Apigenin and Curcumin

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Abstract Apigenin, a less-toxic and non-mutagenic flavonoid, suppressed 12-0-tetradecanoyl-phorbol-13-acetate-(TPA)-mediated tumor promotion of mouse skin. TPA had the ability to activate protein kinase C (PKC) and induced nuclear proto-oncogene expression. Our study indicates that apigenin inhibited PKC by competing with adenosine triphosphate (ATP). Apigenin also reduced the level of TPA-stimulated phosphorylation of cellular proteins and inhibited TPA-induced c-*jun* and c-*fos* expression. Curcumin, a dietary pigment phytopolyphenol, is also a potent inhibitor of tumor promotion induced by TPA in mouse skin. When mouse fibroblast cells were treated with TPA alone, PKC translocated from the cytosolic fraction to the particulate fraction. Treatment with 15 or 20 μM curcumin for 15 min inhibited TPA-induced PKC activity in the particulate fraction by 26–60%. Curcumin also inhibited PKC activity in vitro by competing with phosphatidylserine. Curcumin (10 μM) suppressed the expression of c-*jun* in TPA-treated cells. Fifteen flavonoids were examined for their effects on morphological changes in soft agar and cellular growth in *v*-H-*ras* transformed NIH3T3 cells. The results demonstrated that only apigenin, kaempferol, and genistein exhibited the reverting effect on the transformed morphology of these cells. Based on these findings, it is suggested that the suppression of PKC activity and nuclear oncogene expression might contribute to the molecular mechanisms of inhibition of TPA-induced tumor promotion by apigenin and curcumin. J. Cell. Biochem. Suppls. 28/29:39–48.

Key words: apigenin; curcumin; kaempferol; genistein; PKC; PTK; c-*jun,* c-*fos*

Epidemiological studies have provided convincing evidence that dietary factors can modify the processes of carcinogenesis, including initiation, promotion, and progression of several types of human cancers [1]. Vegetables, fruits, and plants are rich in such polyphenolic compounds as apigenin, curcumin, kaempferol, genistein, catechins, etc. Intensive investigation shows that these phytopolyphenolics are important for maintaining our human health.

One of the functions most widely found in phytopolyphenols of various types is an antioxidant effect based on radical scavenging activity. This function may underlie various effects of polyphenols in plant tissues, and also their pharmacological and medicinal effects related to the inhibition of lipid peroxidation and tumor promotion [2].

Cancer chemoprevention is a means of cancer control in which disease induction as a consequence of exposure to carcinogenic agents can be blocked or reversed by the administration of one or several naturally occurring or synthetic compounds. Among well-studied chemopreventive agents are phytopolyphenols such as apigenin, curcumin, and genistein. The present report summarizes the known inhibitory effects of these phytopolyphenols and their possible molecular mechanisms of action.

ANTI-CARCINOGENIC EFFECT OF APIGENIN

Apigenin (4′,5,7-trihydroxyflavone) (Fig. 1), widely distributed in the plant kingdom, is a less-toxic and non-mutagenic phytopolyphenol

Contract grant sponsor: National Science Council, contract grant number: NSC86-2621-B-002-008-Z; Contract grant sponsor: NHRI Department of Health, contract grant number: DOH86-HR-403.

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Received 12 December 1996; Accepted 8 November 1997

[3–5] and protein kinase inhibitor [6]. The inhibition of protein tyrosine kinase (PTK) by apigenin has been shown to be competitive with respect to adenosine triphosphate (ATP) [6]. Recent studies have shown that apigenin exhibits anti-proliferating effects on human breast cancer cells [7], enhances gap junctional intracellular communication in liver cells [8], and induces morphological changes in some cells [9,10]. The mutagenicity of metabolically activated carcinogen in the Salmonella test system is also inhibited by apigenin [4,11]. In addition, the frequency of sister chromatid exchange in Chinese hamster ovary (CHO) cells is inhibited by apigenin [4]. Furthermore, apigenin has been shown to be an effective inhibitor of B(*a*)P-induced mutagenesis in a hamster embryo cell-mediated V-79 cell mutation assay [12], and to suppress the tumor-promoting effects of 12-0-tetradecanoylphorbol-13-acetate (TPA) on mouse skin initiated with 7,12-Dimethylbenz(*a*)anthracene (DMBA) [13]. The application of apigenin can also suppress TPA-induced epidermal ornithine decarboxylase (ODC) activity [11] and decrease the converting efficiency of papillomas to carcinomas [13].

MECHANISMS OF THE ANTI-CARCINOGENIC EFFECTS OF APIGENIN

Apigenin strongly suppresses TPA-mediated tumor promotion in mouse skin carcinogenesis [13]. This inspired us to investigate the inhibitory effect of apigenin on the partially purified preparation of PKC from NIH3T3 cells. As shown in Figure 2, apigenin inhibited PKC activity dose dependently [14]. Under assay conditions that elicit maximal kinase activity, apigenin inhibited enzymatic activity with an IC_{50} value of 10 ± 0.5 μM. Treatment with 100 ng/ml TPA in NIH3T3 cells at 60 minutes resulted not only in an increase in the amount of 80-KDa phosphoprotein, the useful biomarker of PKC activation in intact cells, but also of other phosphoprotein (Fig. 3). Treatment with 50 and 100 μM apigenin caused a 60 and 80% inhibitory effect on the TPA-stimulated phosphoprotein amount, respectively (Fig. 2), while the total cellular protein remained unaffected by TPA treatment with or without apigenin, as illustrated by silver staining (data not shown). According to the decrease of 80-kDa phosphoprotein, we estimated that IC_{50} of PKC is approximately 40 ± 5 μM in intact cells. Several reports have shown that flavonoids can inhibit tyrosine kinase activity by competing with ATP

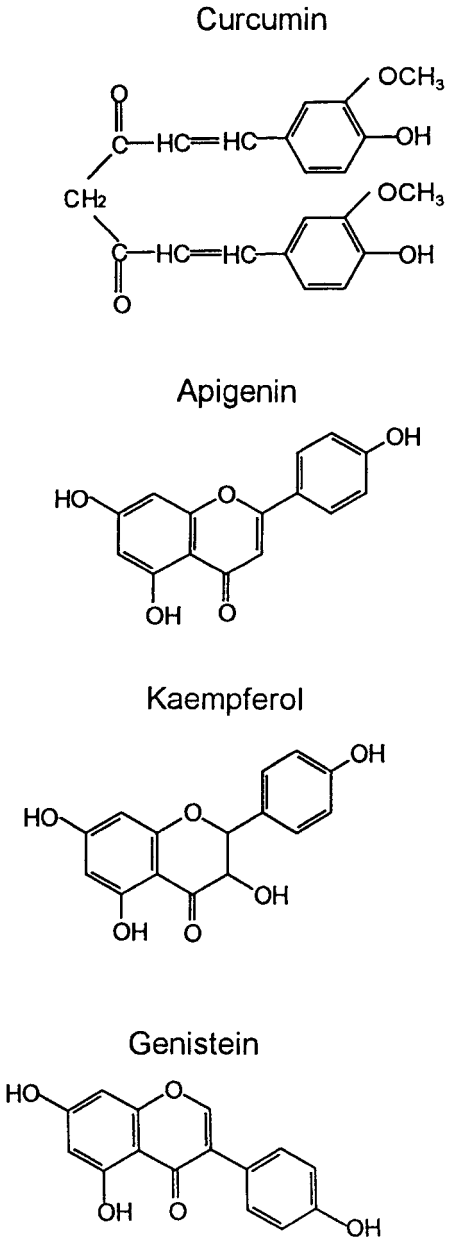

Fig. 1. Structure of curcumin, apigenin, kaempferol, and genistein. These compounds are phytopolyphenols, while apigenin, kaempferol, and genistein are flavonoids.

[6,15]. It is of interest to ascertain the inhibitory mechanism of apigenin on PKC; therefore, experimental kinetic analyses were performed. As shown in Figure 4, the inhibition was competitive with regard to ATP, with a Ki of 0.24 μM in the presence of 10 μM apigenin.

We found that apigenin could inhibit different types of PTK activities. The inhibitory activity of apigenin was more effective in receptor-type PTKs (IC_{50} for EGF receptor, 90 ± 3 μM; for FGF receptor, 20 ± 1.2 μM; and for PDGF

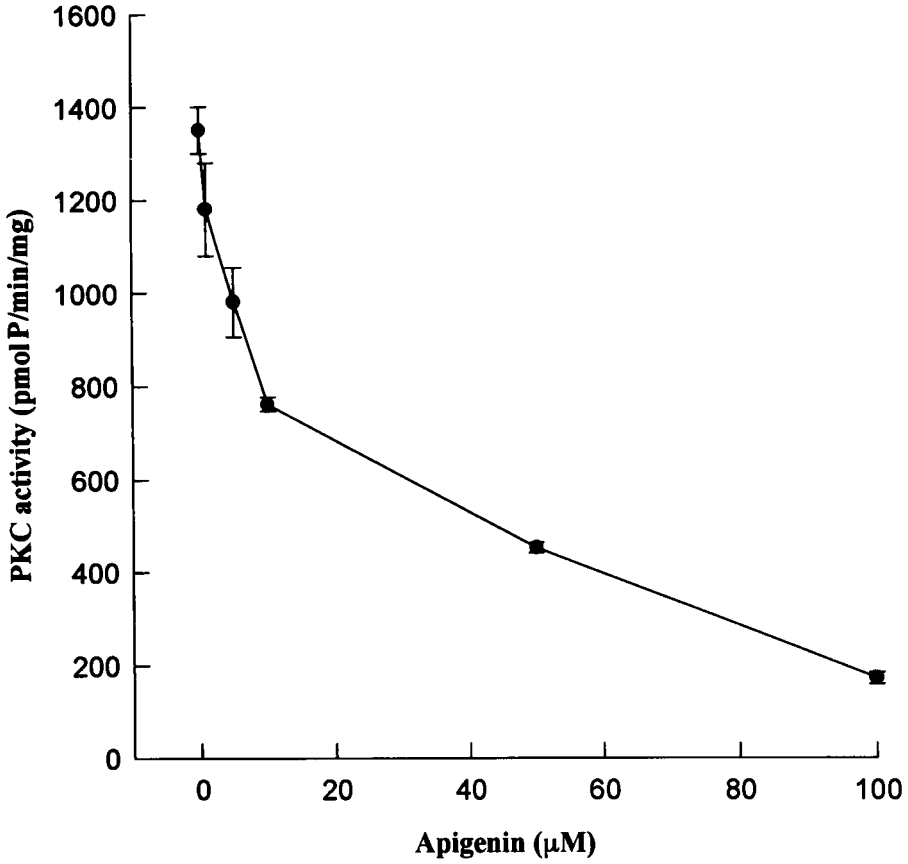

Fig. 2. Effect of apigenin on PKC activity. The activity of partially purified PKC from NIH3T3 was measured as described [14]. Data points are the mean values for 3 independent experiments. Bars represent the S.E.

receptor, 86.6 ± 7.3 μM) than $pp60^{v\text{-}src}$ (IC_{50} 200 μM). In receptor-type PTKs, apigenin showed more selective inhibition of FGF receptor than other receptor-type PTKs. When quiescent NIH3T3 cells were treated with 100 ng/ml TPA, c-*fos* and c-*jun* were transiently induced and reached their maximal levels in 30 and 60 minutes, respectively [16]. The timing of these maximal inductions were selected to study the effects of apigenin on TPA-induced c-*fos* and c-*jun* transcription. Exposure of quiescent cells to 100 ng/ml TPA with 10, 50, and 100 μM apigenin resulted in 50, 80, and 100% suppression of TPA-induced c-*jun* expression, respectively (Fig. 5). Treatment of 100 ng/ml TPA with 10 μM apigenin caused 100% suppression of TPA-induced c-*fos* expression (Fig. 6).

ANTI-CARCINOGENIC EFFECTS OF CURCUMIN

Curcumin (diferuloyl methane) (Fig. 1) is isolated from the rhizoma of the plant *Curcuma longa Linn*. Curcumin has a wide range of biological and pharmacological activities, including antithrombotic effect, antioxidant properties, anti-mutagenic effects, hypocholesterolemic effects in rats, and finally, hypoglycemic effects in man [17].

Curcumin exerts strong anticarcinogenic effects in several organs including skin [18,19], forestomach [19,20], mammary gland [21], colon [20,21], and duodenum [20]. The first report of its anti-tumor effects was published by Huang et al. [18], in which 1, 3, or 10 μM curcumin applied topically to the skin of CD-1 mice inhibited TPA-induced tumor promotion in DMBA-initiated skin. When administrated by gavage at a level of 1 mg per mouse, curcumin reduced the occurrence of benzol(*a*)pyrene (B(*a*)P)-induced forestomach tumors [20]. Subsequent investigations demonstrated the ability of curcumin to inhibit the development of precancerous lesions in DMBA-induced hyperplastic nodules in rat mammary gland tissue [21] and azoxymethane-induced crypts in rat colon [22].

MOLECULAR MECHANISMS OF THE ANTI-CARCINOGENIC EFFECTS OF CURCUMIN

Curcumin inhibited the metabolic activation of B(*a*)P to mutagenic derivative in vitro and the metabolic activation of B(*a*)P to B(*a*)P-DNA

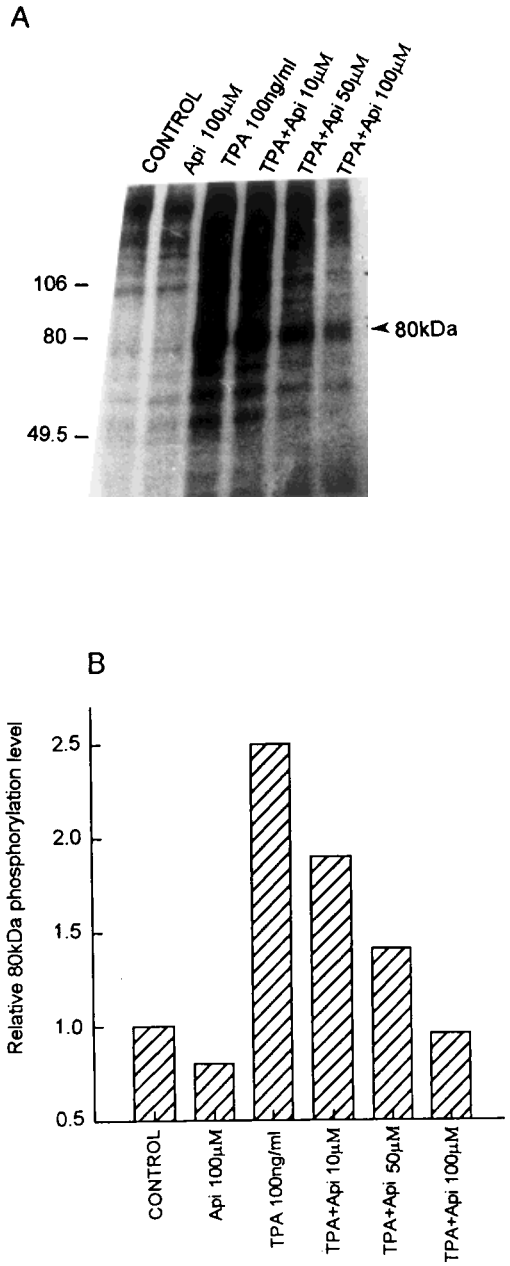

Fig. 3. Inhibition of TPA-induced 80-KDa protein phosphorylation by apigenin in NIH3T3 cells. The TPA-stimulated phosphorylation of 80-KDa protein is shown by an arrowhead, and the positions of molecular weight markers are shown on the left-hand side [14]. **A:** Control, control experiment; TPA: 12-Tetradecanoyl phorbol-13-acetate; Api: Apigenin. **B:** Quantitative integration of A.

adducts in mouse skin in vivo [23,24]. Dietary administration of curcumin to rats or mice was reported to increase the levels of hepatic phase I and phase II enzymes [25].

In the post-initiation phase of carcinogenesis, curcumin has been shown to inhibit TPA-induced ODC activity, cell proliferation, and tumor promotion in mouse epidermis [18]. Curcumin has strong anti-oxidant and free radical scavenging-activity [26], inhibits epidermal arachidonic acid metabolism via lipoxygenase and cyclooxygenase pathways [27], and then inhibits the inflammatory action of arachidonic acid.

The effect of curcumin on a partially purified PKC preparation from NIH3T3 cells is illustrated in Figure 7. The maximal inhibition of PKC by curcumin was obtained at 60 μM [28]. Curcumin did not express its efficiency at a concentration higher than 60 μM because of its water-insolubility. The inhibition was competitive with regard to phosphatidylserine with a Ki of 189 μg/ml in the presence of 30 μM curcumin (Fig. 8). The exposure of intact NIH3T3 fibroblast cells to 0.1 μg/ml TPA caused a redistribution of PKC activity from cytosol to the plasma membrane (particulate fraction). When the cells were treated with 15 μM curcumin and TPA for 15 and 30 minutes, PKC activity was significantly decreased in particulate fraction by 36 and 69%, respectively. However, non-significant decreases in the cytosolic fraction were observed. Treatment with curcumin alone did not significantly change the basal PKC activity. The increased concentration of curcumin enhanced the inhibition of PKC activity on the particulate fraction [28]. However, PKC activity in both partially purified cytosolic and particulate preparations of NIH3T3 cells with or without TPA treatment was inhibited by various concentrations of curcumin in vitro [28].

CURCUMIN INHIBITS THE GENE EXPRESSION OF C-*JUN*

Curcumin at 10, 15, or 20 μM together with TPA at 50 ng/ml was used to treat quiescent cells for 60 minutes. The inhibitory effect of curcumin on the induced c-*jun* mRNA correlated directly with the curcumin dose. Dot hybridization indicated that 10, 15, or 20 μM of curcumin inhibited the TPA-induced increase of c-*jun* mRNA by 21, 28, or 56%, respectively [16]. We also analyzed the effect of curcumin on c-*fos* gene. The product of c-*fos* is thought to be complex and synergistic with c-*jun*/AP-1. In contrast to c-*jun*, however, c-*fos* mRNA was not affected by curcumin after TPA treatment [16].

The TPA product of c-*jun* gene, c-*jun*/AP-1, is a transcriptional factor that functions by binding with a specific enhancer element, TPA-responsive element (TRE). Curcumin (20 μM)

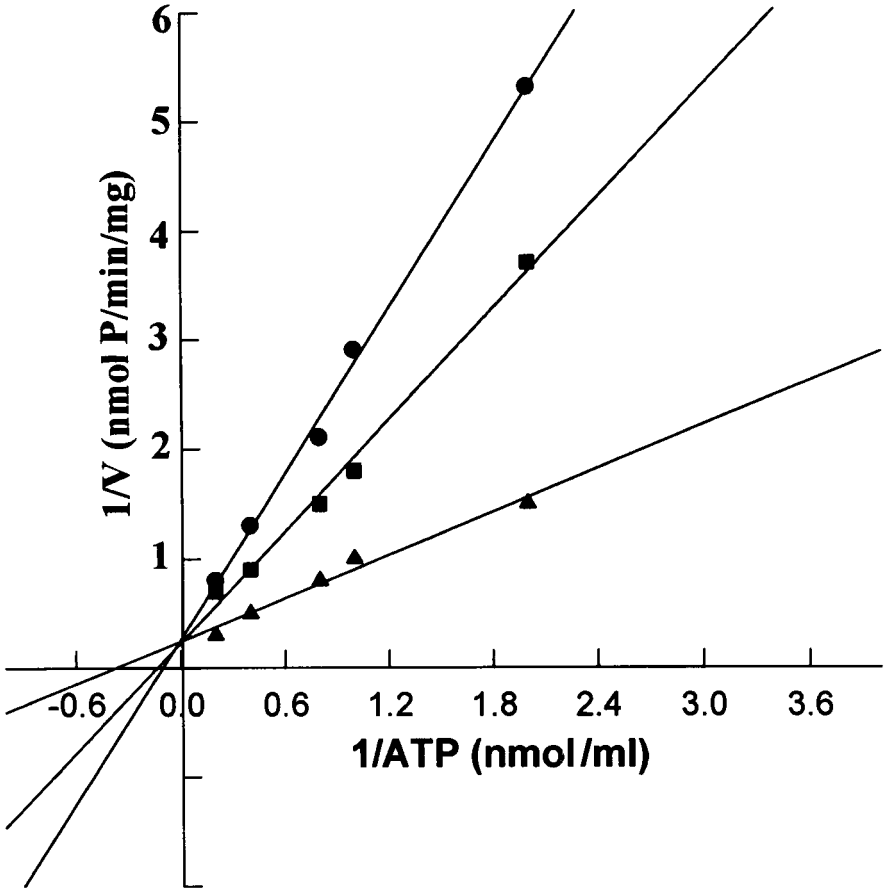

Fig. 4. Competitive inhibition of PKC activity by apigenin. Apigenin 0 (▲), 10 (■), and 50 (●) μM. The inhibition of PKC by apigenin is competitive with respect to ATP (Ki = 0.24 M for 10 μM apigenin) as calculated from double reciprocal plots [14].

could inhibit >50% of TPA-induced TRE-binding activity [16].

We tried to used a transient expression assay to determine the effect of curcumin on TPA-induced transcriptional enhancing activity of the SV40-CAT (pSV2CAT) reporter gene. As expected, 12-hour TPA-induction of NIH3T3 cells transformed with pSV2CAT plasmids enhanced the level of CAT expression 2–3-fold relative to uninduced cells. The TPA-enhanced CAT level was abolished, however, when the transfected cells were treated with TPA plus 10 μM curcumin. The results showed the inhibitory effect of curcumin on TPA-induced transactivating activity of c-*jun*/AP-1.

CURCUMIN INHIBITS THE ACTIVITY OF XANTHINE OXIDASE AND THE FORMATION OF 8-HYDROXYGUANOSINE

Treatment of NIH3T3 cells with the tumor promoter TPA resulted within 30 minutes in a 1.8-fold elevation of xanthine oxidase activity, an enzyme capable of generating reactive oxygen species such as superoxide and hydrogen peroxide. Simultaneous administration of 2 and 10 μM curcumin with 100 ng/ml TPA inhibited TPA-induced xanthine oxidase activity measured 30 minutes later by 22.7 and 36.5%, respectively [29]. We have demonstrated that the hydroxylation of deoxyguanosine in a hydroxyl free radical-generating system is significantly inhibited by curcumin (10 μM). The TPA-induced formation of 8-hydroxyguanosine and lipid peroxidation in NIH3T3 cells is remarkably suppressed by the presence of curcumin [30].

APIGENIN AND OTHER FLAVONOIDS REVERT THE TRANSFORMED PHENOTYPES OF *V*-H-*RAS* NIH3T3 CELLS

Fifteen flavonoids (25–75 μM) were examined for their effects on the morphology of *v*-H-*ras* NIH3T3 cells (Table I). Among these flavonoids, only three compounds—apigenin, kaempferol, and genistein (Fig. 1)—exhibited a reverting effect on the transformed phenotype at the concentration of 25 μM [31]. After treatment with these three compounds, the refractive and overlapping morphology of transformants were changed into flatter, polygonal, and

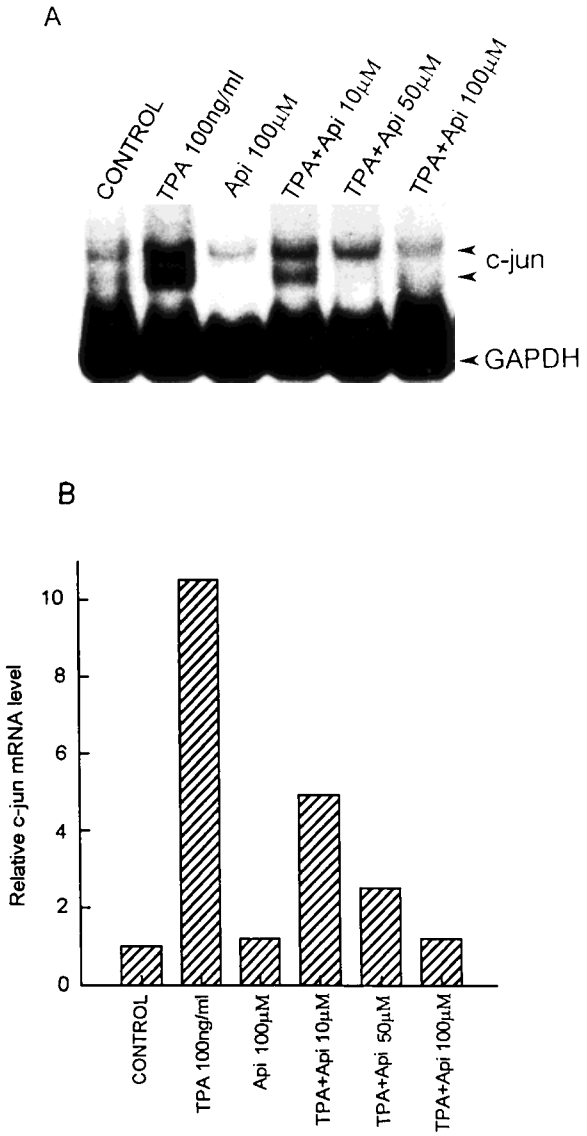

Fig. 5. Suppression of TPA-induced c-*jun* mRNA expression in NIH3T3 cells by apigenin. **A:** Arrowheads indicate c-*jun* or GAPDH mRNA. Results were performed by three independent experiments, only one representative is shown [14]. See Figure 3 for definitions. **B:** Quantitative integration of A.

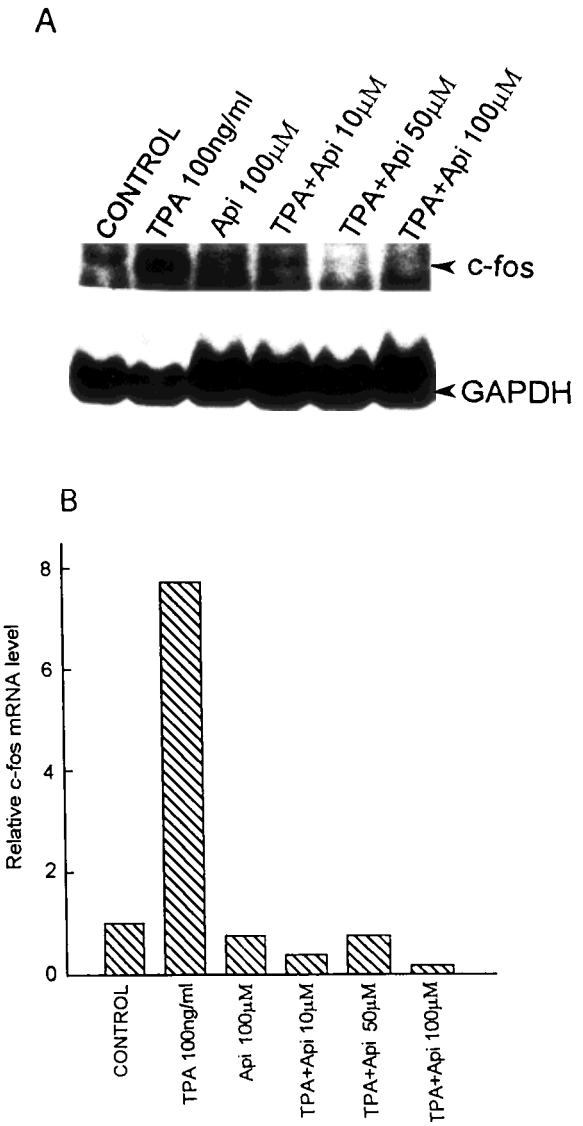

Fig. 6. Suppression of TPA-induced c-*fos* mRNA expression in NIH3T3 cells by apigenin. **A:** Arrowheads indicate c-*fos* or GAPDH mRNA. Results were performed by three independent experiments; only one representative is shown [14]. See Figure 3 for definitions. **B:** Quantitative integrations of A.

less phase-dense cells within 24 hours. The morphological alternations were dose-dependent.

Anchorage-independent growth (colony formation in soft agar), another criteria for transforming activity, was also used to examine the reversional potency of these 15 flavonoids. Table I shows that 25 μM of apigenin, kaempferol, and genistein significantly inhibited the *v*-H-*ras* transformant growth in soft agar, the effective dose range being consistent with that which induced the morphological change; the other compounds were inactive even at concentrations up to 75 μM [31].

CURCUMIN-INDUCED APOPTOSIS IN IMMORTALIZED NIH3T3 AND MALIGNANT CANCER CELL LINES

We have demonstrated that curcumin (30–90 μM) induces such characteristics of apoptosis as cell shrinkage, chromatin condensation, and DNA fragmentation in immortalized mouse embryo fibroblast, NIH3T3, *erb*B2 oncogene-transformed NIH3T3, mouse sarcoma S180, human

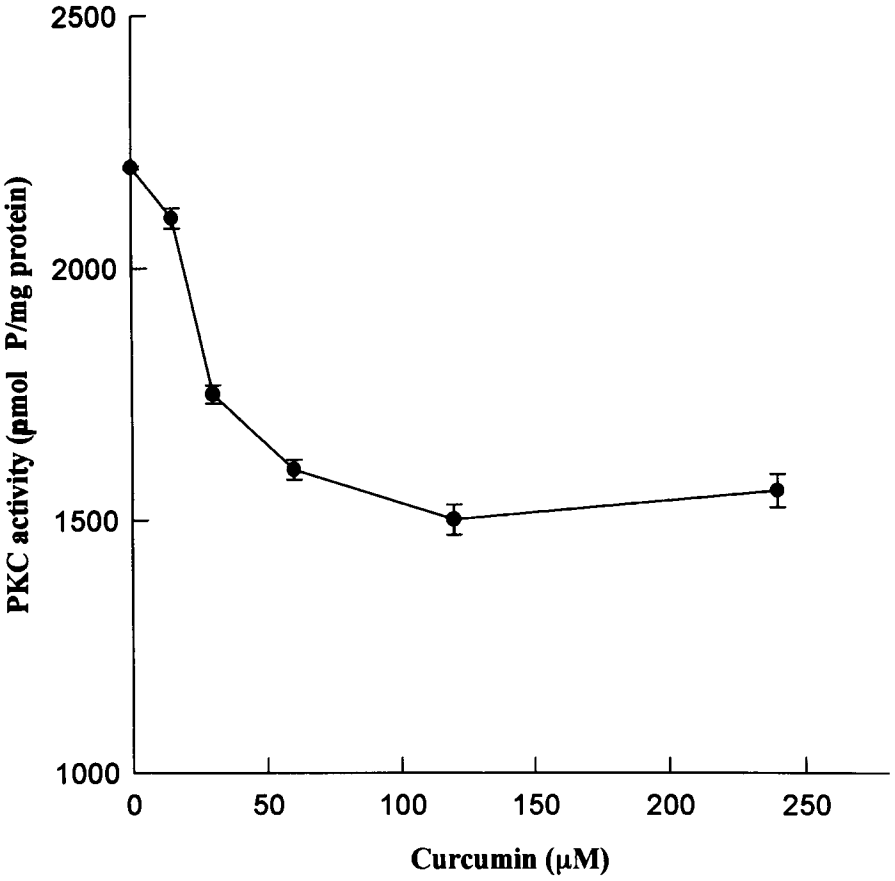

Fig. 7. Effect of curcumin in PKC activity. PKC partially purified from NIH3T3 cells was measured as described [28]. The enzyme (4.1 μg protein/50 μl) was incubated for 3 minutes in the presence of 10 μg phosphatidylserine, 0.75 μg 1,2 diolein. 0.35 μmol $CaCl_2$, and concentrations of curcumin as indicated. Values of four independent experiments [±SE (bar)].

colon cancer cell HT29, human kidney cancer cell 293, and human hepatocellular carcinoma HepG2 cells, but not in primary culture of mouse embryonic fibroblast C3H10T1/2, rat embryonic fibroblast, and human foreskin fibroblast cells in a concentration- and time-dependent manner [32]. Treatment of NIH3T3 cells with the PKC inhibitor staurosporine, the tyrosine kinase inhibitor herbimycin A, and the arachidonic acid metabolism inhibitor quinacrine induces apoptosis. These results suggest that, in immortalized and transformed cells, blocking the cellular signal transduction might trigger the induction of apoptosis.

DISCUSSION ON THE ACTION MECHANISMS OF CURCUMIN, APIGENIN, AND FLAVONOIDS

During the course of cell proliferation in carcinogenesis, numerous oncogenes are expressed, possibly as intermediates in the signal transduction pathways. In the cascade of molecular activity through which hormones—and growth factors that regulate cell growth, proliferation, and differentiation—communicate across cell membranes, signal transduction involves intermediary molecules known as second messengers. The evidence for oncogene activity in signal transduction in carcinogenesis is based on the similarity of some products (protein kinase) to other intermediates [33]. Some recent investigations demonstrate that compounds that inhibit oncogene activation also inhibit carcinogenesis [34].

One of the steps in signal transduction involves activation of the enzyme PKC by diacylglycerol (DAG). There is evidence that carcinogenesis may be suppressed by inhibiting this enzyme. Structurally, the tumor promoter TPA can replace DAG in activating PKC. Chemicals that inhibit PKC, such as D,L-palmitoylcarnitine, flavonoids, 18β-glycyrrhetinic acid, and N-(6-aminohexyl)-5-chloro-1-1 naphthalene-sulfonamide (W-7), also inhibit TPA-induced tumor promotion in mouse skin.

Intensive studies on the action of curcumin and apigenin in various biological systems have indicated that these compounds have engaged in multiple anti-tumor promoting pathways. It has been demonstrated that TPA-induced tu-

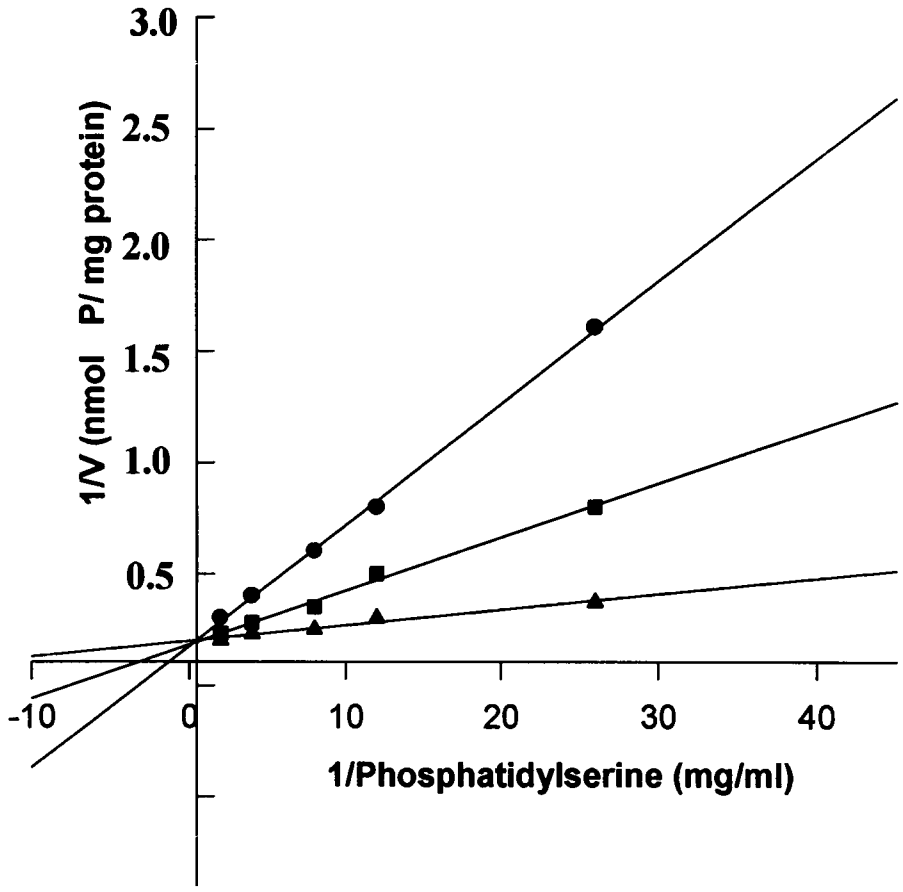

Fig. 8. Competitive inhibition of activity by curcumin. The enzyme was assayed under standard conditions [28] except for varying concentrations of phosphatidylserine as indicated and curcumin [0 (▲), 30 (■), 60 (●) μM]. The inhibition was competitive with respect to phosphatidylserine (Ki = 189 μg/ml for 30 μM curcumin) in the absence of 1,2 diolein, as calculated from double reciprocal plot.

mor promotion is effectively inhibited by curcumin [18,20,25]. TPA, a versatile, biologically active agent, induces several biosynthetic processes, namely enhanced expression of cellular oncogenes such as c-*jun*, c-*fos*, and c-*myc*, induction of ornithine decarboxylase, elevation or translocation of PKC, induction of cyclooxygenase and lipoxygenase, and others. It seems that all of these biochemical processes are required for anabolic pathways and cell proliferation. It is noteworthy that all of these processes have been effectively inhibited by the presence of curcumin, apigenin, or other flavonoids (Fig. 1).

Two mechanisms prevent the ongoing process of carcinogenesis: reversion of neoplastic transformation (by differentiation), and induction of apoptosis. With these aspects, the reversion of the transformed phenotypes of *v*-H-*ras* NIH3T3 cells by apigenin, kaempferol, and genistein (Table I) is particularly interesting and deserves further investigation. A recent study on the action mechanism of apigenin by Lepley and Pelling has demonstrated that apigenin treatment of human diploid fibroblasts produces a G1 cell-cycle arrest by inhibiting cdk2 kinase activity and the phosphorylation of Rb and inducing the cdk inhibitor p21/WAF1, all of which may mediate its chemopreventive activities in vivo [35]. On the other hand, the present finding that curcumin induces apoptosis in several malignant cancer cell lines as well as several immortalized cell lines may provide a new dimension in assessing this compound as a promising chemopreventing agent.

It is apparent that the molecular mechanisms of action of curcumin, apigenin, and other flavonoids are quite complicated and multiple. The action of these compounds may proceed simultaneously or sequentially from DNA (gene) level to RNA and protein (enzyme) levels. Accordingly, we propose the following pathways for actions of curcumin, apigenin, and related flavonoids. The primary target could be on the plasma membrane where the activities of PKC or receptor tyrosine kinases are first inhibited. Some kinase-mediated nuclear protein factors are then inhibited through various signal transduction mechanisms.

TABLE 1. Effects of Apigenin and Other Flavonoids on the Anchorage-Independent Growth in *v*-H-*ras*-Transformed NIH3T3 Cells

Flavonoids	Concentration (μM)	Colony formation[a]	Morphological reversion[b]
Control	–	0.92	–
Flavone	75	0.93	–
Apigenin	25	0.11	+
Chrysin	75	0.90	–
Genistein	25	0.32	+
Quercetin	75	0.63	+
Hesperetin	75	0.88	–
Epicatechin	75	0.93	–
Kaempferol	25	0.15	+
Diosmin	75	0.89	–
Diamide	75	0.88	–
Lucigenin	75	0.87	–
Myricetin	75	0.91	–
Rutin	75	0.90	–
Luteolin	75	0.85	–
Daidzein	75	0.90	–

[a]Colony formation is expressed as a ratio of the growth of the flavonoid-treated cells to that of *v*-**H-***ras* 3T3 control cells. The average value for growth of *v*-**H-***ras* 3T3 cells in soft agar was 920 ± 53 colonies/dish or 92%, expressed as percentage of the number of cells originally plated in soft agar (1×10^5).

[b]Cultures were considered to be transformed (–) or reverted (+) on the basis of morphological observations conducted at 7 days after treating with tester compounds at the indicated concentrations. Transformed cells were spindle-shaped and grew in an overlapping meshwork; reverted cells, in general, were flat, polygonal, and grew in monolayers. In this study, three concentrations namely 25, 50, and 75 μM were used. It seemed that 75 μM was the maximum tolerable dose for this cell line.

ACKNOWLEDGMENTS

This study was supported by the National Science Council (NSC86-2621-B-002-008-Z) and NHRI Department of Health (DOH86-HR-403).

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