Pergamon

European Journal of Cancer Vol. 32A, No. 1 pp. 146–151, 1996

Printed in Great Britain
0959-8049/96 $15.00+0.00

0959-8049(95)00540-4

## Original Paper

# Inhibitions of Protein Kinase C and Proto-oncogene Expressions in NIH 3T3 Cells by Apigenin

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**Apigenin, a low-toxic and non-mutagenic plant flavonoid, suppresses 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-mediated tumour promotion of mouse skin. TPA has the ability to activate protein kinase C (PKC) and induce proto-oncogene expression. Our study shows that apigenin inhibits PKC by competing with ATP, and exhibits an $IC_{50}$ value of $10 \pm 0.5$ μM. Apigenin also reduces the level of TPA-stimulated phosphorylation of cellular proteins. Of the protein tyrosine kinases tested, the fibroblast growth factor (FGF) receptor was most strongly affected by apigenin ($IC_{50}$ 20 μM), and $pp60^{v\text{-}src}$ most weakly affected ($IC_{50} > 200$ μM). Treatment of NIH 3T3 cells with 100 ng/ml TPA and 10, 50 and 100 μM apigenin resulted in 50, 80 and 100% suppression of TPA-induced *C-JUN* expression, respectively. Treatment of TPA with 10 μM apigenin inhibited TPA-induced *C-FOS* expression. TPA-stimulated cell growth was suppressed by 25 μM apigenin. Our results provide some evidence for understanding apigenin's inhibitory effects of TPA-mediated tumour promotion.**

**Key words: protein kinase C, TPA, *C-FOS*, *C-JUN*, apigenin, protein tyrosine kinases**
*Eur J Cancer*, Vol. 32A, No. 1, pp. 146–151, 1996

## INTRODUCTION

TUMOUR PROMOTION is a complex and important process in carcinogenesis, generally studied using 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) promotion in mouse skin models [1]. In the past, TPA treatment has resulted in activation of protein kinase C (PKC) [2, 3], increase of ornithine decarboxylase (ODC) activity [4], induction of immediate–early gene expression [5, 6], increase of totally cellular phosphoprotein amount [7, 8], induction of active oxygen species generation [9, 10], and an increase in the amount of 8-hydroxydeoxyguanosine [10, 11]. These phenomena may form useful biomarkers for TPA-induced tumour promotion; and, based on current knowledge of TPA-mediated signal transduction, PKC activation by TPA is essential [12]. PKC activity in intact cells can be evaluated by analysing the phosphorylation level of endogenous 80-kDa proteins, which are specific substrates of PKC [7, 8]. When PKC activity is induced by TPA, several cellular proteins are phosphorylated directly or indirectly, leading to the induction of some proto-oncogenes, such as *C-FOS* and *C-JUN* [12].

Apigenin (4,5,7-trihydroxyflavone) is a low-toxic and non-mutagenic plant flavonoid [13–16] and a protein kinase inhibitor [17, 18]. The inhibition of protein tyrosine kinase (PTK) by apigenin has been shown to be competitive with respect to ATP [17]. Recent studies have shown that apigenin exhibits antiproliferating effects on human breast cancer cells [19], induces morphological changes in some cells [20, 21], and enhances gap junctional intercellular communication in liver cells [22]. The mutagenesis of metabolically activated carcinogens in the *Salmonella* test system is also inhibited by apigenin [4, 15, 23]. In addition, Kuo and colleagues found that apigenin could reduce the frequency of sister chromatid exchange in Chinese hamster ovary cells [15]. Apigenin has also been shown to be an effective inhibitor of benzo[a]pyrene-induced mutagenesis in a hamster embryo cell-mediated V79 cell mutation assay [24] and to suppress the tumour-promoting effects of TPA on mouse skin initiated with 7,12-dimethylbenz[a]anthracene (DMBA) [25]. The application of apigenin can also suppress TPA-induced epidermal ODC activity [4] and decrease the converting efficiency of papillomas to carcinomas [25].

In the present study, we investigate apigenin inhibition of PKC and PTKs activity. Apigenin suppressed TPA-induced *C-FOS* and *C-JUN* expression, and TPA-stimulated cell growth. Our results also provide some evidence toward understanding apigenin's inhibitory effect on TPA-mediated tumour promotion.

## MATERIALS AND METHODS

### *Chemicals*

Phenylmethylsulphonyl fluoride (PMSF), leupeptin, histone H1 (type III-s), phosphatidylserine, 1,2-diolein (1,2-

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Revised 16 Aug. 1995; accepted 23 Aug. 1995.

dioleoyl-rac-glycerol), sucrose, ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol, Tris (hydroxymethyl)-amino-methane hydrochloride (Tris–HCl), TPA, formamide, formaldehyde, sodium dodecyl sulphate (SDS), poly (Glu, Na, Tyr) 4:1 ($Glu_4Tyr$) and apigenin were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). The random primer labelling system was ordered from Oncogene Science (Uniondale, New York, U.S.A.). [$\gamma^{32}$P]ATP (5000 Ci/mmol), [$^3$H]thymidine (25 Ci/mmol) and [$\alpha^{32}$P]dCTP (3000 Ci/mmol) were obtained from Amersham (Buckinghamshire, U.K.). BCA protein assay reagent was purchased from Pierce (Rockford, Illinois, U.S.A.). TPA was dissolved in absolute ethanol. Apigenin was dissolved in diluted-potassium hydroxide doubly-distilled water (pH 9.8).

*Cell culture*

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Seromed, Berlin, Germany) containing 10% fetal calf serum (FCS) (Seromed, Germany), and antibiotics (100 U/ml penicillin, 100 U/ml steptomycin) (Biological Industries, Israel). For quiescent cells, 80% confluent cells were serum-starved in 0.5% FCS DMEM for 24–36 h before various treatments.

*Protein phosphorylation and analysis of phosphoprotein*

Protein phosphorylation analysis was modified from that outlined by Rodriguez-Pena and colleagues [7]. Briefly, NIH 3T3 cells ($5 \times 10^5$ cells/ml) were grown in a six-well plate overnight, and then serum-starved for 24–36 h. The cells were further incubated in phosphate-free DMEM containing $^{32}$Pi (200 μCi/ml) for 3 h at 37°C. TPA, with or without apigenin, was added directly to the medium, then incubated for another 30 min. The reaction was terminated by rapidly washing the cells with ice-cold Tris/saline solution (0.15 M $CaCl_2$/20 mM Tris–HCl pH 7.5) three times. Cells were incubated with 5% TCA for 10 min twice, and then rinsed twice with ice-cold Tris/saline solution. Cells were treated with 200 μl lysis buffer (10 mM Tris–HCl pH 7.5/2 mM EDTA/2 mM PMSF/3% SDS/0.1% NP-40/10 mM NaCl/0.15 mM $MgCl_2$). Cellular lysates were centrifuged at 14000*g* at 4°C for 10 min. The supernatants were collected and the protein contents were determined by using the BCA protein assay reagent with bovine serum albumin as a standard. Each sample, which contained 50 μg protein, was separated on a 10% SDS–polyacrylamide gel [26]. After electrophoresis, gels were stained, dried and autoradiographed (X ray film Kodak XRA-5) with one intensifying screen at −70°C. Relative intensity of $^{32}$P-labelled 80-kDa bands were scanned using a densitometer (Zeineh, Model SLR-2D/1D, Biomed Instruments, Fullerton, California, U.S.A.).

*Probes*

A 1-kb *Pst*I insert of *PFOS-1* [27], an *Eco*RI fragment of a full-length *C-JUN* coding sequence [28] and a GAPDH cDNA *Pst*I fragment cut from pIBI30GAPDH [29] were used. These probes were all labelled with [$\alpha^{32}$P]dCTP using a random primer labelling system.

*Total RNA isolation and Northern blot analysis*

NIH 3T3 cells were grown in 10-cm dishes to approximately 80% confluence, and were then serum-starved for 24–36 h. TPA, with and without apigenin, was added directly to the medium for 30 and 60 min, the cells were then harvested for RNA extraction. Total RNA was isolated as described by Chomczynski and Sacchi [30]. For Northern blots, 25 μg of total RNA per lane was fractionated by electrophoresis on a 1.2% agarose gel containing 6.7% formaldehyde. RNA was transferred to Zeta-probe Nylon filters (Bio-Rad, Richmond, California, U.S.A.), incubated, prehybridised and hybridised for 12–16 h at 42°C in hybridisation buffer (6 × SSC (sodium chloride and sodium citrate), 50% deionised formamide, 10 × Denhart's solution, 10 mM EDTA, 0.1% SDS) containing approximately $1 \times 10^6$ cpm/ml of probes [31]. The filters were then washed successively in 2 × SSC, 0.1% SDS; 0.5 × SSC, 0.1% SDS; 0.1 × SSC, 0.1% SDS for 15 min in each solution at room temperature. Filters were then exposed to X-ray film (Kodak XAR-5) between two intensifying screens at −70°C. The X-ray films were developed and scanned in order to estimate the density of the bands.

*PKC activity measurement*

PKCs were partially purified from quiescent NIH 3T3 cells [32]. Briefly, cells were washed twice with ice-cold phosphate-buffered saline (PBS) (0.125 M NaCl, 10 mM $Na_2HPO_4$, 3 mM $KH_2PO_4$ pH 7.2) and twice with buffer A (20 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.3 M sucrose, 50 mM 2-mercaptoethanol, 2 mM PMSF and 10 μg/ml leupeptin). The cells were scaped, homogenised and centrifuged at 100 000*g* for 1 h at 4°C. The supernatant was applied to an ion-exchange column containing 1 ml of diethyl aminoethyl (DEAE)-cellulose (Sigma DE52) equilibrated with buffer B (20 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF). The column was washed four times with 3 ml buffer B and 1 ml buffer B containing 30 mM potassium chloride. PKC activity was assayed by the transfer of $^{32}$P-labelled phosphate for ATP to histone as previously described [32, 33]. The incubation mixture (0.2 ml) contained 50 μl (2–10 μg) of the partially purified PKC sample, 5 μmol Tris–HCl pH 7.4, 2 μmol $MgCl_2$, 40 μg of lysine-rich histone, 10 μg of phosphatidylserine, 0.75 μg of 1,2-diolein, 10 μg/ml leupeptin, 5 μM [$\gamma^{32}$P]ATP (4000 cpm/pmol), and presence/absence of 0.35 μmol $CaCl_2$. The mixture was incubated at 30°C for 3 min, and terminated by addition of 1 ml of ice-cold 20% (w/v) trichloroacetic acid. Kinase activity was expressed as pmol of Pi incorporated per min per mg protein of purified sample.

*Thymidine incorporation assays*

NIH 3T3 cells ($2.5 \times 10^5$) were grown on 10-cm dishes overnight and were then serum-starved in 0.5% FCS for 24 h. The cells were treated with TPA for 24 h in the presence or absence of the various doses of apigenin, followed by addition of [$^3$H]thymidine 10 μCi/ml. Treated cells were washed twice with ice-cold PBS, twice with 5% trichloroacetic acid, air-dried, dissolved in 1 N NaOH and assayed for radioactivity as described by Kumar and colleagues [34].

*Protein tyrosine kinase assays*

Isolation of membrane protein was modified from Chao and colleagues [35]. In brief, cells were washed twice with ice-cold PBS, scraped and homogenised in buffer A (10 mM HEPES pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The homogenate was centrifuged. The pellet was resuspended in buffer A containing 0.5% Triton X-100 at 4°C for 30 min, then centrifuged 10 000*g* at 4°C for 10 min, and

the supernatant was used for kinase assays. The standard kinase assay mixture (final volume 60 μl in 50 mM HEPES buffer pH 7.4) contained 10 μg of membrane protein, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 100 μM ATP, 10 μM [$\gamma^{32}$P]ATP, 0.67 mg/ml poly $Glu_4Tyr$, 1 μg/ml different ligands [fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF)] and the various doses of apigenin. The mixture was incubated for 20 min at 22°C, and the reaction was terminated by adding 20 μl stop solution (120 mM EDTA, 4 mM $Na_3VO_4$). Fifty microlitres of the terminated mixture were spotted on to Whatman P81 paper (Whatman International Ltd, Maidstone, U.K.) (2 × 2 $cm^2$) followed by three washings with 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. The filters were air-dried and analysed for radioactivity as described previously [36, 37].

## RESULTS

Wei and colleagues reported that apigenin strongly suppresses TPA-mediated tumour-promoting effects on mouse skin [25]. As TPA is an activator of PKC, we explored whether apigenin could inhibit PKC activity. We studied the inhibitory effect of apigenin using a partially purified preparation of PKC from NIH 3T3 cells. As shown in Figure 1, apigenin inhibited PKC activity dose-dependently. Under assay conditions which 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 NIH 3T3 cells at 60 min resulted not only in an increase in the amount of 80-kDa phosphoprotein, a useful biomarker of PKC activation in intact cells, but also of other phosphoproteins (Figure 2, TPA). As TPA treatment caused an increase in the amount of total cellular phosphoprotein, we examined whether apigenin could suppress the increase in the amount of phosphoprotein stimulated by TPA. Treatment with 50 and 100 μM apigenin caused a 60 and 80% inhibitory effect on the TPA-stimulated phosphoprotein amount, respectively (Figure 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 the $IC_{50}$ of PKC is approximately 40 ± 5 μM in intact cells. Several reports have shown that flavonoids can inhibit kinase activity by competing with ATP [17, 18, 38]. To ascertain the inhibitory mechanism of apigenin on PKC, therefore, detailed kinetic analyses were performed. As shown in Figure 3, the inhibition was competitive with regard to ATP, with a $K_i$ of 0.24 μM for 10 μM apigenin. We also determined whether apigenin could inhibit PTK activity. As

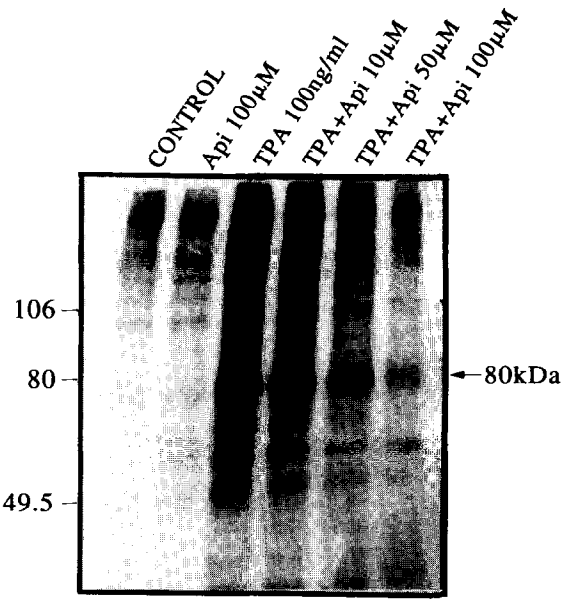


**Figure 2. Inhibition of TPA-induced 80-kDa protein phosphorylation by apigenin in NIH 3T3 cells. The TPA-stimulated phosphorylation of the 80-kDa protein is shown by an arrow, and the positions of molecular weight markers are shown on the left-hand side. Results were obtained from three independent experiments, only one representative is shown here. Symbols: CONTROL, control experiment; TPA 100 ng/ml, 100 ng/ml TPA-treated only; Api 100 μM, 100 μM apigenin-treated only; TPA + Api 10 μM, TPA + Api 50 μM and TPA + Api 100 μM, treated with 10, 50 and 100 μM apigenin with 100 ng/ml TPA, respectively.**

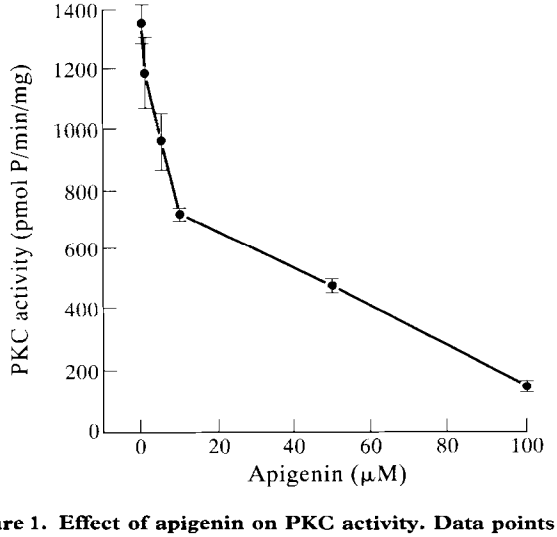


**Figure 1. Effect of apigenin on PKC activity. Data points are the mean values for three independent experiments. Bars represent the S.E.**

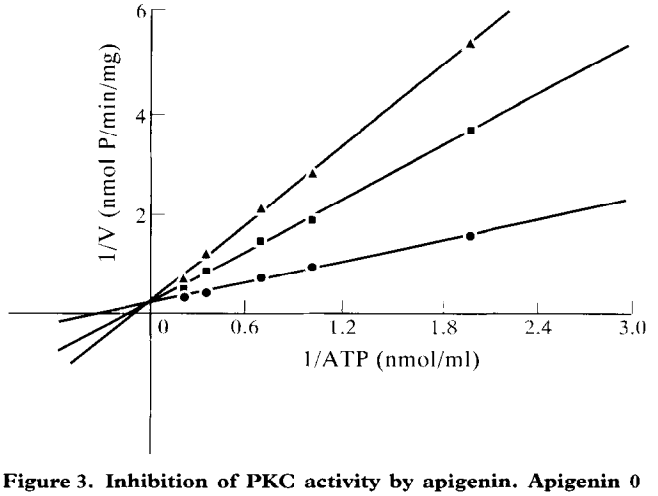


**Figure 3. Inhibition of PKC activity by apigenin. Apigenin 0 (●), 10 (■) and 50 (▲) μM. The inhibition of PKC by apigenin is competitive with respect to ATP ($K_i$ = 0.24 μM for 10 μM apigenin) as calculated from double reciprocal plots. Each point represents the mean of three independent experiments, in which duplicate determinations were made.**

*Table 1. Effects of apigenin on the protein tyrosine kinase (PTK) activities*

| Types of PTK | $IC_{50}$ (μM)* |
|---|---|
| EGF receptor | 90 ± 3 |
| FGF receptor | 20 ± 1.2 |
| PDGF receptor | 86.6 ± 2.3 |
| $pp60^{v\text{-}src}$ | >200 |

*The $IC_{50}$ was determined from the inhibition curve taking probit of inhibition per cent at varied concentrations of apigenin on abscissa and logarithm of concentrations of apigenin on ordinate. Each experiment was independently performed three times. EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor.

shown Table 1, we found that apigenin could inhibit different types of PTK activities. The inhibitory activity of apigenin was more effective in receptor-type PTKs ($IC_{50}$ 20–90 μM) than $pp60^{v\text{-}src}$ ($IC_{50}$ >200 μM). In receptor-type PTKs, apigenin showed more selective inhibition of FGF receptor ($IC_{50}$ 20 ± 1.2 μM) than other receptor-type PTKs.

TPA can stimulate the transient expression of *C-FOS* and *C-JUN*, which are proto-oncogenes, so we investigated whether apigenin could suppress TPA-induced *C-FOS* and *C-JUN* transcription. When quiescent NIH 3T3 cells were treated with 100 ng/ml TPA, *C-FOS* and *C-JUN* mRNAs were transiently induced and reached their maximal levels in 30 and 60–90 min, respectively [39]. The timing of 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 (Figure 4). Treatment of 100 ng/ml TPA with 10 μM apigenin caused 100% suppression of TPA-induced *C-FOS* expression (Figure 5). Finally, we tested whether apigenin could inhibit TPA-induced cell proliferation. Table 2 shows that apigenin alone had no significant effect on cell survival, but could inhibit stimulation of [$^3$H]thymidine incorporation into DNA in TPA-treated cells.

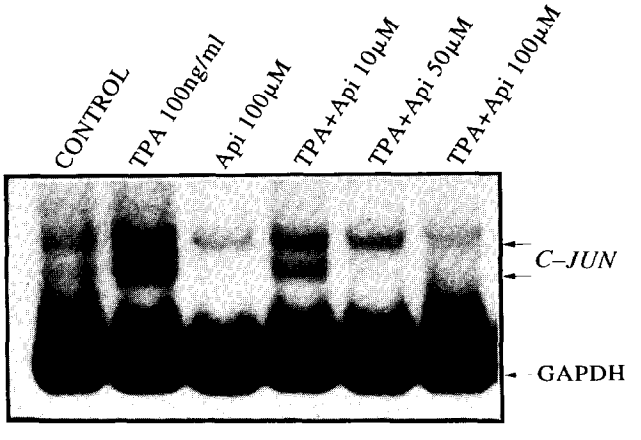


**Figure 4. Suppression of TPA-induced *C-JUN* mRNA expression in NIH 3T3 cells by apigenin. Arrows indicate *C-JUN* or GAPDH mRNAs. Results were performed by three independent experiments, only one representative is shown. See Figure 2 legend for label definitions.**

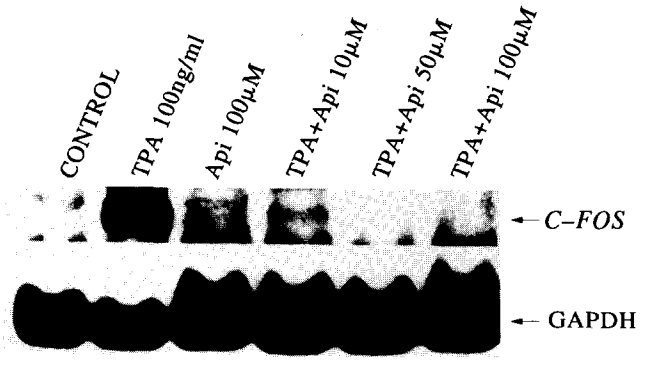


**Figure 5. Suppression of TPA-induced *C-FOS* mRNA expression in NIH 3T3 cells by apigenin. Arrows indicate *C-FOS* or GAPDH mRNA. Results were performed by three independent experiments, only one representative is shown. See Figure 2 legend for label definitions.**

*Table 2. Effects of apigenin on TPA-stimulated [$^3$H]thymidine incorporation in NIH 3T3 cells*

| Treatment | [$^3$H]Thymidine incorporation (relative ratio to control) |
|---|---|
| Control | 1 |
| TPA | 1.77 |
| Apigenin (25 μM) | 0.97 |
| TPA + apigenin (10 μM) | 1.51 |
| TPA + apigenin (15 μM) | 1.43 |
| TPA + apigenin (25 μM) | 1.01 |

Each experiment was independently performed three times. TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

## DISCUSSION

Apigenin is a plant flavonoid that occurs naturally and widely in fruits and vegetables, such as celery, and is one of the important constituent flavonoids in human daily diet [13, 16]. Apigenin can inhibit carcinogenesis in rodents [25] and is considered to have a potential role in the prevention of human cancer. Human carcinogenesis is known to proceed through multiple stages, such as initiation, promotion and progression [40]. Tumour promotion, which is a complex and important process in carcinogenesis, is generally studied using TPA mouse skin models [1]. Apigenin has the ability to suppress TPA-mediated tumour-promoting effects on mouse skin [25]. PKC is a primary cell receptor for TPA [2, 3]. Our results showed that apigenin can inhibit PKC activity. It is a competitive inhibitor of ATP binding, and its mechanism of inhibition is similar to that of other flavonoids [17, 18, 38]. However, we found that the $IC_{50}$ concentration (approximately 40 ± 5 μM) in intact cells was higher than in partially purified PKC ($IC_{50}$ 10 ± 0.5 μM). The different $IC_{50}$ values may have been caused by apigenin having to penetrate cells to block PKC's ATP binding. Thus, the intact cell's $IC_{50}$ value would be expected to be higher than in partially purified PKC. In the future, we will use a PKC-specific substrate to investigate the inhibitory effects of apigenin on the $Ca^{+2}$-dependent/independent activities.

We also found that apigenin inhibited PTK activity. The $IC_{50}$ concentration of the EGF receptor was 90 ± 3 μM, similar to other observations [18] and our data showed that the FGF receptor had the lowest $IC_{50}$ concentration (20 ± 1.2 μM).

Thus, the inhibitory effects of apigenin appear to be more specific for PKC than for other kinases, such as PTKs and protein kinase A [17, 18]. Therefore, apigenin may be a useful biochemical tool to investigate PKC function. TPA treatment resulted not only in an increase in 80-kDa phosphoproteins, but also of other phosphoproteins between 80 and 40 kDa. Because TPA can simulate the generation of active oxygen species, which can activate some protein kinases, including PKC [41, 42], the increase of phosphoprotein stimulated by TPA may result from TPA-generated active oxygen species and activation of PKC. Alternatively, apigenin has been found to work as an active oxygen species scavenger [43, 44]. Therefore, that apigenin inhibited the TPA-stimulated phosphoproteins may be attributed to its inhibition of PKC as well as its active oxygen species scavenging activity. It may well be that the inhibitory effects of apigenin on PKC activity and TPA-stimulated phosphorylation will prove to have inhibitory effects on tumour formation.

The gene expression which can be transiently induced by TPA is one of the events that trigger the onset of tumour promotion. The number of known inducible genes has grown to at least 30 [45, 46], of which *C-FOS* and *C-JUN* are two of the immediate–early proto-oncogenes closely related to cell growth and transformation [47–50]. Interestingly, it has been reported that, while the induction of both genes greatly increases in early stages, it gradually declines in the late stages of two-step carcinogenesis in C3H/10T1/2 cell models [5]. According to these reports, over-induction of the two proto-oncogenes by TPA in the early stages of tumour promotion may be one of the events leading to carcinogenesis [5, 12]. Conversely, agents which inhibit the expression of these proto-oncogenes may act in chemoprevention. In this study, we demonstrated that apigenin could suppress TPA-induced *C-FOS* and *C-JUN* expression. Furthermore, apigenin also inhibited TPA-stimulated cell proliferation. Therefore, apigenin might work as a chemopreventive agent in the mouse skin model [25], but for humans further study is needed.

The two species of *C-JUN* transcripts observed in this study have been reported by Ryder and Nathans [48] and are accounted for by the different positions of poly(A) addition signals. The larger is more stable than the smaller. Apigenin inhibition of PKC caused differential disappearance rates in the two *C-JUN* transcripts. The difference may result from an unequal stability of two transcripts. We also found that the suppression of TPA-induced *C-FOS* transcription by apigenin was at least 10-fold greater than that of *C-JUN* expression. These results indicated that the induction of these two proto-oncogenes is somewhat different and that *C-FOS* expression is more susceptible to apigenin than is *C-JUN*. We compared calphostin C, chelerythrine chloride and staurosporine with apigenin, and found that these could only inhibit TPA-induced *C-FOS*, but not *C-JUN* (data not shown). This observation is similar to another study [51].

According to the current TPA-mediated signal transduction model, our data suggest that the suppression of *C-FOS* and *C-JUN* expression by apignenin may contribute to the inhibition of PKC activity. However, when we compared the inhibition of the dose–response curve of PKC activity with proto-oncogene expression, we found that the dose needed to inhibit PKC activity in intact cells (approximately $40 \pm 5$ μM) by 50% was higher than that needed to inhibit 50% of the proto-oncogene expression (*C-FOS* $IC_{50} < 10$ μM; *C-JUN* $IC_{50}$ $10 \pm 2.5$ μM). The different concentrations imply that TPA-induced *C-FOS* and *C-JUN* expression may not be simply mediated by PKC. Recently, many studies have shown that active oxygen species could stimulate *C-FOS* as well as *C-JUN* expression in various cell systems [52–55]. Many studies reported that TPA could stimulate the generation of active oxygen species *in vivo* [9, 10, 56, 57]. Our studies showed that induction of *C-JUN* expression by hydrogen peroxide, an active oxygen species, was decreased by down-regulated PKC (data not shown). This experimental data suggest that TPA-induced *C-FOS* and *C-JUN* expression may be a result of both active oxygen species and PKC. Therefore, the differentially inhibitory concentrations may result from apigenin's active oxygen species-scavenging activity as well as its inhibitory effect on PKC.

Cell proliferation is a very complex process, including *C-FOS* and *C-JUN* expression in early G1. When some of the proliferation signals are impaired, it may result in prevention or decrease in cell proliferation. Apigenin 25 μM can completely inhibit *C-FOS* and partially suppress *C-JUN* induction and PKC activity. These may cause the prevention of TPA-stimulated cell proliferation. Many reports have shown that active oxygen species play an important role in tumour promotion, including that which is TPA-mediated (see review [56, 57]). Semba and Inui reported that generation of active oxygen species accompanied by activation of PKC is essential to the TPA-mediated promotion process [58]. Therefore, the active oxygen species-scavenging activity of apigenin may result in its anti-TPA tumour promotion and in the different dose required for PKC inhibition and suppression of cell proliferation.


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**Acknowledgements**—This work was supported by grants NSC 81-0421-B002-535 (M-L Kuo) and NSC 82-0421-B002-202 (J-K Lin) from the National Science Council and NRICM-82109 (J-K Lin) from the National Research Institute of Chinese Medicine, Taipei, Taiwan, Republic of China.