0026-895X/96/020249-09$3.00/0

MOLECULAR PHARMACOLOGY, 50:249–257 (1996).

Inhibition of 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Ornithine Decarboxylase Activity by Genistein, a Tyrosine Kinase Inhibitor

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Received February 19, 1996; Accepted April 8, 1996

SUMMARY

The effects of the protein tyrosine kinase inhibitor genistein on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity in monkey kidney epithelial CV-1 cells were determined. CV-1 cells were pretreated with genistein for 2 hr before treatment with 100 nM TPA. ODC activity was determined 9 hr after TPA treatment. Genistein inhibited TPA-induced ODC activity at 0.1, 1, 10, 25, 50, 100, 200, and 400 μM by 0%, 0%, 42%, 59%, 62%, 81%, 91%, and 100%, respectively (IC_{50} = 20 μM). Genistein inhibited TPA-induced mitogen-activated protein kinase (MAPK) tyrosine phosphorylation and the accumulation of steady state levels of ODC mRNA at 400 μM but not at 25 μM. Genistein, at 25 μM, did not alter the TPA-induced phosphorylation of p90 ribosomal S6 kinase but caused a ~50% decrease of the TPA-induced phosphorylation of p70 S6 kinase (p70S6K), a protein kinase involved in the control of translational efficiency. Taken together, these data indicate that genistein may inhibit TPA-induced ODC activity at the transcriptional and translational levels through the inhibition of MAPK and p70S6K activation, respectively. The regulation of MAPK and p70S6K may be mediated through different protein tyrosine kinases that have differential sensitivity to genistein.

TPA is a potent mouse skin tumor promoter (1). The phosphorylation of cellular proteins and the transcription and/or translation rates of growth-related genes are significantly increased after TPA treatment (2–5). ODC, a cellular protein that plays a key role in cell proliferation, is dramatically induced by TPA in mouse skin, newborn mouse epidermal cells, T24 cells, and CV-1 cells (3, 4, 6, 7). Transgenic mice overexpressing ODC proteins are more sensitive than nontransgenic mice to tumor promotion by TPA, although the spontaneous tumor indices do not increase (8). Evidence also indicates that overexpression of ODC, by cooperating with other oncogenic proteins (i.e., c-H-*ras*), induces the cellular transformation in NIH 3T3 cells (9). It has also been postulated that ODC may act as an oncogene because expression of ODC from appropriate plasmid vectors leads to a transformed phenotype (10). On the other hand, chemopreventive agents, such as retinoic acid and α-difluoromethylornithine, which inhibit TPA-induced ODC activity, also reduce tumor formation (11–13). These studies suggest that ODC is an important target to prevent cell transformation.

Expression of TPA-induced ODC activity is regulated at multiple levels, including the transcriptional rate of the ODC gene, and the translational efficiency of ODC mRNA (6, 14–17). Recently, we isolated a human ODC gene from a human leukocyte genomic DNA library and narrowed the TPA-responsive sequence to 96 bp between −42 bp and +54 bp relative to the transcriptional initiation site (15). Interestingly, this ODC gene fragment does not contain any consensus AP-1 binding sequence, a major TPA-responsive element found in several TPA-inducible genes. Also, binding of the HeLa cell nuclear proteins to this fragment could not be completely competed with 100-fold molar excess of AP-1, AP-2, or AP-3 sequences (15). Furthermore, the activation of the TPA-responsive ODC luciferase reporter construct (−42/+54-ODC-luc) could not be blocked by the expression of dominant negative c-*jun*.[1] These results indicate that the activation of ODC transcription by TPA may involve a different signal transduction pathway than used for the activation of AP-1 and AP-1 regulated genes.

Translational control of ODC mRNA also plays a key role

This work was supported by National Institutes of Health Grant CA35368.

[1] C.-P. Tseng and A. K. Verma, unpublished observations.

ABBREVIATIONS: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; SAMD, *S*-adenosyl-L-methionine decarboxylase; MAPK, mitogen-activated protein kinase; p90RSK, p90 ribosomal S6 kinase; p70S6K, p70 S6 kinase; ECL, enhanced chemiluminescence; PI, propidium iodide; AP, activator protein 1; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

in ODC gene expression. Two factors may contribute to influence the translatability of the ODC mRNA. A long and highly conserved 5′-untranslated region of the ODC mRNA has been shown to form an extensive, stable secondary structure that may control the rate of translation (14, 17–19). The presence of "minicistrons" (short open reading frame sequences) in the upstream regions of the translational initiation sites has been suggested to inhibit the initiation of translation by interfering with the "scanning" of ribosomal initiation complexes (20, 21). The translational control of the ODC mRNA is not fully defined in the TPA-treated cells. Several studies suggest that TPA induces the phosphorylation and activation of p90RSK and p70S6K, which by activation may phosphorylate ribosomal S6 protein and subsequently increase translational efficiency (22–25). Recently, repression of p70S6K by rapamycin has been shown to decrease the translation of pyrimidine-rich mRNA (26). These results indicate that in addition to the structure of the mRNA, some other components in the translational machinery may be modulated by TPA that may increase the expression of certain genes.

Protein kinase C activity has been shown to be involved in the regulation of TPA-induced ODC activity (4, 6, 16). However, the downstream events in the transcriptional and/or translational control of TPA-induced ODC activity followed by PKC activation are not completely understood. Protein phosphorylation at tyrosine residues is a key component in the regulation of eukaryotic cell growth and differentiation. Mitogens, such as EGF, serum, insulin, and TPA, have been shown to increase the cellular phosphotyrosine contents (27, 28). One of the phosphotyrosine proteins that has been demonstrated to be linked to many cellular events induced by mitogens is MAPK (29, 30). MAPK, also known as ERK, is a family of protein kinases that require phosphorylation at both serine/threonine and tyrosine residues for activation (31). Activated MAPK phosphorylates and regulates protein kinases involved in the regulation of gene expression (32).

Protein tyrosine kinase inhibitors are useful agents with which to dissect the signaling pathway involving protein tyrosine phosphorylation (33–35). With the property of inhibiting cell growth, they are also potential therapeutic agents for cancer treatment (36–38). Genistein, an isoflavone isolated from soy milk and soy food, is a protein tyrosine kinase inhibitor (39). Genistein inhibits the growth of NIH 3T3 cells and a number of tumor cell lines *in vitro* (40–43). Genistein also inhibits the induction of ODC activity by gastrin in colonic mucosa (34).

We determined the effect of genistein on TPA-induced ODC activity in CV-1 cells. We further analyzed the possible mechanisms of action of genistein, which may provide clues about the mechanisms of TPA-induced ODC activity. We present data indicating that genistein may inhibit TPA-induced ODC activity at both transcriptional and translational levels, involving the inhibition of MAPK and p70S6K activation, respectively. The regulation of MAPK and p70S6K may be mediated through different protein tyrosine kinases that have differential sensitivities to genistein.

Experimental Procedures

Materials. CV-1 cells and 18S RNA probe were purchased from American Type Culture Collection (Rockville, MD); anti-pan MAPK antibody and antiphosphotyrosine antibody PY20 were purchased from Transduction Laboratories (Lexington, KY); p90RSK and p70S6K antibodies and protein A/G plus agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); cell culture medium and reagents were purchased from GIBCO (Grand Island, NY); GeneScreen nylon membrane, [α-^{32}P]dCTP, [^{14}C]ornithine hydrochloride, and *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine were purchased from New England Nuclear (Boston, MA); the random primer kit was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); genistein was purchased from LC Laboratories (Woburn, MA); ECL reagents were purchased from Amersham (Arlington Heights, IL); and TPA was purchased from Life Systems (Newton, MA). All other chemicals were of reagent grade.

ODC activity assay. Monkey kidney epithelial CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated at the density of 10^6/10-cm dish. Confluent cells were starved in serum-free medium for 48 hr. At the indicated time after treatment, cells were harvested, and cell pellets were resuspended in homogenization buffer containing 50 mM Tris·HCl, pH 7.5, 0.1 mM pyridoxal-5-phosphate, and 0.1 mM EDTA. After freeze/thaw and centrifugation, the ODC activity in the soluble extracts was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride (44).

SAMD activity assay. Cell extracts were prepared in ODC homogenization buffer as described above. The reaction mixture consisted of 0.5 μmol of putrescine and 3.4 nmol of *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine (0.2 μCi/assay) in a final volume of 0.2 ml. The reaction was stopped by the addition of 0.5 ml of 2 M citric acid. Enzyme activity was determined by measuring the release of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine (45).

Cell viability assay. Flow cytometric analysis of cell viability was done as described previously (46). Briefly, after an indicated time of treatment, cells were detached with 0.25% trypsin/EDTA solution. Approximately 10^6 cells were resuspended in 1 ml of PBS. Ten microliters of PI (1 mg/ml) was added to the cell suspension, and the PI uptake was analyzed by flow cytometry. Cell viability was represented by the percentage of PI-exclusive cells.

RNA isolation and Northern blot analysis. Total cellular RNA was prepared by the guanidinium thiocyanate phenol/chloroform method (47). The indicated amount of total RNA was fractionated on a 1% agarose-formaldehyde gel. The RNA was transferred to a GeneScreen Nylon membrane using 20× SSC (1X SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) as transfer buffer. After hybridization, the ODC cDNA probe (pODC10/2H) was labeled with [α-^{32}P]dCTP by the random primer method (48) and then added to the hybridization solution at a final specific activity of 10^6 cpm/ml. At 18 hr after hybridization, the membrane was washed twice with 2× SSC/0.1% SDS at room temperature for 20 min and once with 0.1× SSC/0.1% SDS at 65° for 20 min. To determine the equal loading of RNA on the gel, the membrane was reprobed with [α-^{32}P]dCTP-labeled 18*S* RNA as an internal control. Quantification was accomplished with a soft laser densitometer.

Immunoprecipitation. After the indicated treatment, cells were washed twice with cold PBS. For MAPK, cells were scraped into 450 μl of lysis buffer A (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 0.01 M sodium fluoride, and 1 mM EGTA) (49). Cells were transferred to 1.5-ml Eppendorf tubes and kept on ice for 30 min. After centrifugation, the supernatants were used as total cell lysates. For p70S6K, cells were scraped into 450 μl of lysis buffer B (10 mM potassium phosphate, pH 6.5, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM vanadate, 50 mM β-glycerophosphate, 0.1% Triton X-100, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin) and homogenized (50). After centrifugation, the supernatants were used as total cell lysates. The immunoprecipitation reaction (50 μl of protein A/G plus agarose, 450 μl of cell lysates, and 5 μg of antibody) was performed at 4° for

2 hr. After three washes with radioimmunoprecipitation assay buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) or lysis buffer B (for p70S6K), the pellets were dissolved in 1× SDS sample buffer (60 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.01% bromophenol blue) for Western blot analysis.

Western blot analysis. The expression of proteins was detected by the ECL method with some modifications (51). Briefly, cell pellets were disrupted either directly in the 1× SDS sample buffer (for MAPK) or in lysis buffer B (for p90RSK and p70S6K). Cell extracts were fractionated on a 7.5% (for p90RSK and p70S6K) or 10% (for MAPK) SDS-polyacrylamide gel. After electrotransfer to a nitrocellulose membrane, the nonspecific binding sites were blocked by immersing the membrane into blocking buffer (1% bovine serum albumin in 10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) overnight at room temperature. On the following day, the membrane was incubated with antibody diluted in wash buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 hr. After being washed for 30 min, the horseradish peroxidase-labeled secondary antibody was added and incubated for 1 hr at room temperature. After the final wash, the detection reagents were mixed and applied to cover the surface of the membrane. For autoradiography, film was exposed as described by the manufacturer. Quantification was accomplished with soft laser densitometry.

Results

Genistein inhibits TPA-induced ODC activity. We first determined the time of treatment for maximal inhibition of TPA-induced ODC activity by genistein. In this experiment, 25 μM genistein was added to confluent CV-1 cells either 2 hr before, concurrent with, or 3 or 6 hr after TPA treatment. Cells were harvested to determine ODC activity at 9 hr after TPA treatment. Genistein, added either 2 hr before or concurrent with TPA, inhibited TPA-induced ODC activity by 67% (Fig. 1A). The inhibition of TPA-induced ODC activity was only 20% when genistein was added 6 hr after TPA treatment.

The effects of different concentrations of genistein on TPA-induced ODC activity in CV-1 cells are shown in Fig. 1B. Genistein was added to the confluent cells at 2 hr before TPA treatment. The cells were harvested to determine ODC activity at 9 hr after TPA treatment. As shown in Fig. 1B, genistein inhibited TPA-induced ODC activity with an IC_{50} value of ~20 μM. The ODC activity was dramatically reduced to 30% of control (DMSO/TPA-treated cells) with 50 μM genistein. However, 400 μM genistein was required to completely inhibit TPA-induced ODC activity. The inhibition of TPA-induced ODC activity by treatment with 25 μM genistein was specific because genistein at 25 μM did not inhibit TPA-induced SAMD activity, another enzyme in polyamine biosynthesis (Fig. 1C).

Effect of genistein on cell viability. To determine whether the inhibition of TPA-induced ODC activity by genistein is due to its cytotoxic effect, a cell viability assay using PI uptake was performed. We found no significant difference in cell viability between cells treated with TPA alone or TPA plus different concentrations of genistein (Table 1). All treatments contained >90% viable cells. We further analyzed the uptake of Hoechst 33342 using flow cytometry to determine whether genistein induced apoptosis. No significant apoptotic cell death was observed between different treatments (data not shown).

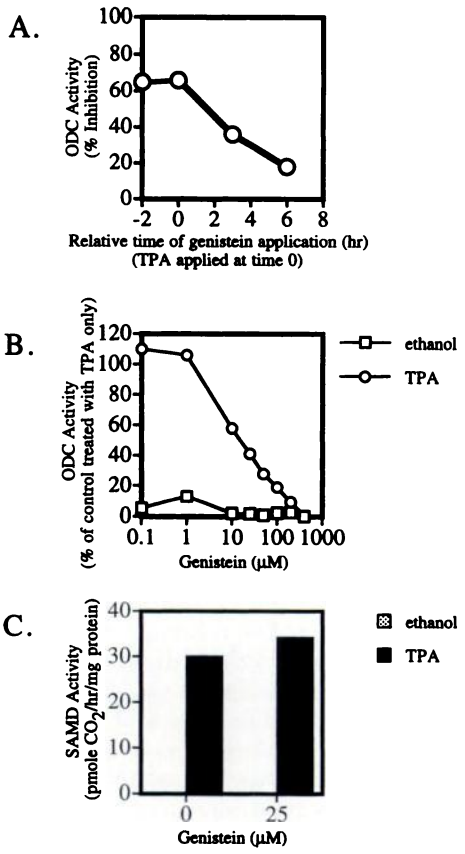

Fig. 1. Effect of genistein on TPA-induced ODC activity and SAMD activity. A, Treatment time of genistein. CV-1 cells were plated at the density of 10^6/10-cm culture dish. Confluent cells (~3 days after plating) were serum-starved for 48 hr and incubated with 25 μM of genistein 2 hr before, concurrent with, or 3 or 6 hr after TPA treatment. After 9 hr of TPA treatment, cells were harvested and assayed for ODC activity. Data represent the percentage of inhibition of ODC activity relative to DMSO/TPA-treated cells. Each data point is the average of duplicate assays (variations < 10%). B, Dose effects of genistein. Cell culture conditions were as described in A. CV-1 cells were pretreated with DMSO or varying concentrations of genistein (0.1, 1, 10, 25, 50, 100, 200, and 400 μM) for 2 hr. TPA (final concentration = 100 nM) or ethanol (final concentration = 0.01%) was added directly to the medium for an additional 9 hr. Cells were harvested in ODC homogenization buffer to assay ODC activity as described in Experimental Procedures. The ODC activity is plotted as the percentage of ODC activity in CV-1 cells treated with the DMSO/TPA. Each data point is the mean ± standard error of five to seven experiments. C, Effects of genistein on SAMD activity. Cell extracts were prepared after treatment of CV-1 cells with genistein and TPA as described in B. SAMD activity was assayed as described in Experimental Procedures. Data represent the average of duplicate assays (variation <10%). Similar results were obtained in two repeated experiments.

Genistein inhibits TPA-induced ODC mRNA. The question of whether the inhibition of TPA-induced ODC activity correlates with the inhibition of TPA-induced ODC mRNA level by genistein was explored. In this experiment, at the indicated time after TPA treatment, RNA was isolated, and the level of ODC mRNA was determined by Northern blot analysis. A human ODC cDNA probe (pODC10/2H) was used to detect the ODC transcript. TPA treatment caused an ~5-fold increase in steady state levels of ODC mRNA. The TPA-induced ODC mRNA level was not significantly altered when CV-1 cells were pretreated with genistein ≤200 μM (Fig. 2, A and B). Treatment with a higher concentration of

TABLE 1

Cell viability after treatment of CV-1 cells with genistein and TPA

Confluent, serum-starved CV-1 cells were pretreated with the indicated concentrations of genistein for 2 hr followed by ethanol (0.01% v/v) or TPA (100 nM) treatment for 9 hr. Cells were trypsinized to prepare a single cell suspension. Then, 10 μl of PI (1 mg/ml) was added to 1 ml of cell suspension (~10^6 cells/ml) for analysis by flow cytometry. We analyzed 5000 cells for each treatment. The cell viability was represented as the percentage of PI exclusive cells. Data represent the mean of duplicate assays.

Genistein	Cell viability	
	Ethanol	TPA
μM	% PI exclusive cells	
0	95	96
25	94	94
50	93	96
100	95	94
200	95	92
400	92	93

genistein (400 μM) resulted in the inhibition of TPA-induced accumulation of ODC mRNA (Fig. 2, A–C).

Effect of genistein on TPA-induced tyrosine phosphorylation. To obtain clues about the role of tyrosine phosphorylation in TPA-induced ODC activity, we determined the effect of genistein on TPA-induced tyrosine phosphorylation. A time course of TPA-induced tyrosine phosphorylation is shown in Fig. 3A. In this experiment, confluent CV-1 cells were treated with ethanol vehicle (0.01%) or 100 nM TPA. At the indicated time (1 min, 5 min, 10 min, 30 min, 1 hr, and 9 hr) after treatment, cells were lysed directly in 1× SDS sample buffer for Western blot analysis. The phosphotyrosine proteins were detected by the antiphosphotyrosine antibody PY20. As shown in Fig. 3A, two proteins at molecular mass of ~42 and ~44 kDa were preferentially tyrosine phosphorylated by TPA treatment (Fig. 3A). The phosphorylation occurred at 10 min after TPA treatment, whereas maximum phosphorylation was observed at 30 min after TPA treatment.

To determine whether genistein inhibited the TPA-induced tyrosine phosphorylation of the 42- and 44-kDa proteins, we pretreated confluent CV-1 cells for 2 hr with different concentrations of genistein. Cells were harvested 30 min after TPA treatment to detect phosphotyrosine proteins by Western blot analysis. Genistein, at 400 μM but not at <200 μM, decreased the level of TPA-induced tyrosine phosphorylation of the 42- and 44-kDa proteins (Fig. 3, B and C). A noteworthy observation is the finding that the tyrosine phosphorylation of 42- and 44-kDa proteins, and not of the other major phosphortyrosine proteins, was specifically inhibited by 400 μM genistein.

Effect of genistein on TPA-induced tyrosine phosphorylation of MAPK. TPA induces tyrosine phosphorylation of p42MAPK and p44MAPK, the major MAPK isoforms (52). To determine whether the 42- and 44-kDa tyrosine-phosphorylated proteins (Fig. 3) were MAPK, we immunoprecipitated the cell lysate with antiphosphotyrosine antibody. The immunocomplex was resolved on SDS-polyacrylamide gel electrophoresis. Tyrosine phosphorylation of MAPK was determined by Western blot analysis using an anti-pan MAPK antibody, which recognizes MAPK-related proteins of molecular masses 42, 44, 54, and 85 kDa (53). As shown in Fig. 4A, TPA treatment increased the tyrosine phosphorylation content of 42- and 44-kDa MAPK proteins.

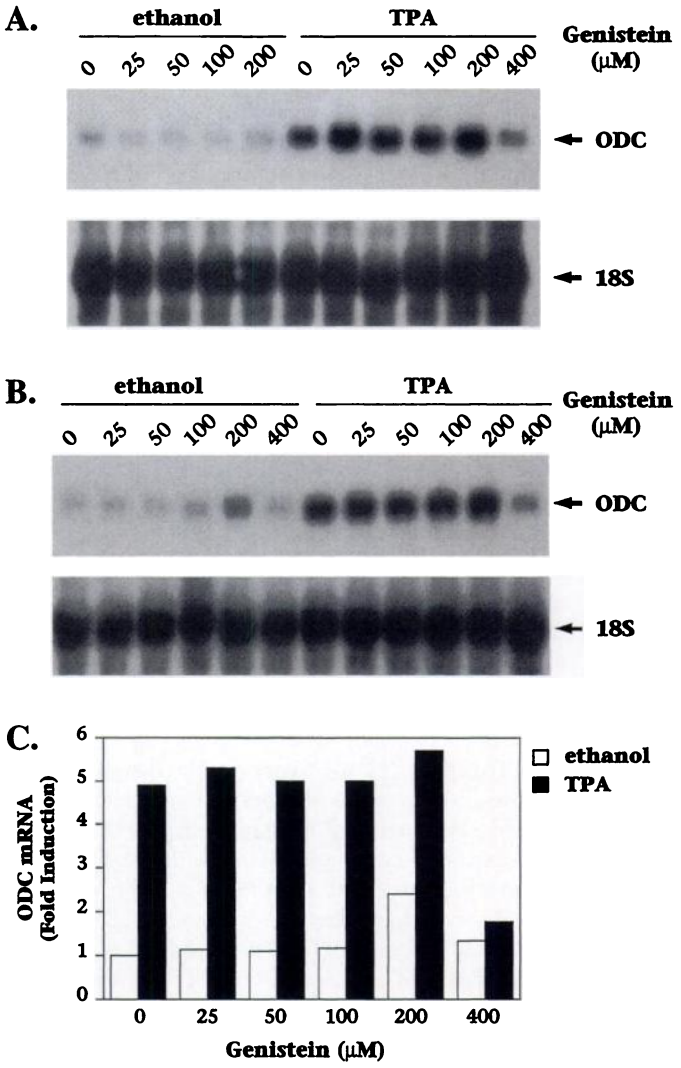

Fig. 2. The effect of genistein on TPA-induced ODC mRNA. A and B, Northern blot analysis (A and B represent two independent experiments). The cell culture and treatment condition of genistein and TPA are the same as described in the legend for Fig. 1. At 9 hr after treatment, total cellular RNA was isolated. Then, 10 μg of total RNA was fractionated on a 1% agarose gel. The ODC transcript was detected by a randomly primed ^{32}P-labeled ODC cDNA probe, pODC10/2H. 18S RNA was used as the internal control for equal loading. C, Quantification of the Northern blot analysis was done with a soft laser densitometer. The expression of ODC mRNA in ethanol/DMSO-treated cells was arbitrarily set at 1. Data represent the average value of experiments shown.

We further determined whether genistein inhibited TPA-induced tyrosine phosphorylation of MAPK. We pretreated confluent CV-1 cells with different concentrations of genistein followed by 30-min TPA treatment. Cells were harvested, and a mobility shift assay with anti-pan MAPK antibody was performed. As shown in Fig. 4B, 400 μM genistein inhibited the hyperphosphorylation of MAPK proteins as elicited by the disappearance of the slowest mobility shift band.

Effect of genistein on TPA-induced phosphorylation of p90RSK and p70S6K. The protein kinases p90RSK and p70S6K have been shown to phosphorylate ribosome S6 protein *in vitro* and are implicated in the translational control of mRNA *in vivo* (22–25). Because translational control of ODC

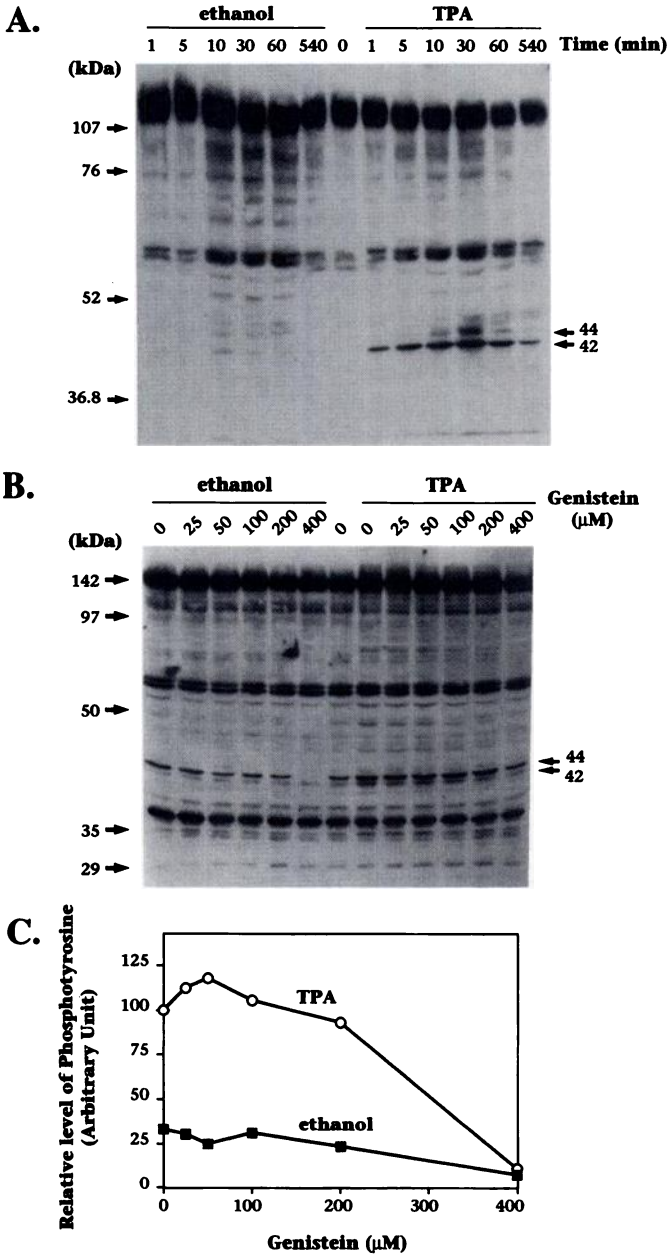

Fig. 3. Effect of genistein on TPA-induced tyrosine phosphorylation in CV-1 cells. A, Time course of TPA-induced tyrosine phosphorylation. CV-1 cells were cultured as described in the legend for Fig. 1. After treatment with 100 nM TPA or ethanol for the indicated time, cells were lysed in 1× SDS sample buffer. Equal amounts of protein (100 μg) were fractionated on a 7.5% SDS-polyacrylamide gel. The phosphotyrosine proteins were detected with the antiphosphotyrosine antibody PY20 coupled with ECL detection. B, Effect of genistein on TPA-induced tyrosine phosphorylation. CV-1 cells were cultured as described in the legend for Fig. 1. After pretreatment with the indicated concentration of genistein (0, 25, 100, 200, and 400 μM), cells were treated with 100 nM TPA or ethanol for 30 min. CV-1 cells were lysed directly with 1× SDS sample buffer. One fifth of the cell lysates (100 μl) were fractionated on a 10% SDS-polyacrylamide gel. The phosphotyrosine proteins were detected with the antiphosphotyrosine antibody PY20 coupled with ECL detection. C, Quantification of the phosphorylation level of the 42/44-kDa proteins in Fig. 3B was done with a soft laser densitometer. Data are expressed as the level of tyrosine phosphorylation relative to DMSO/TPA-treated cells.

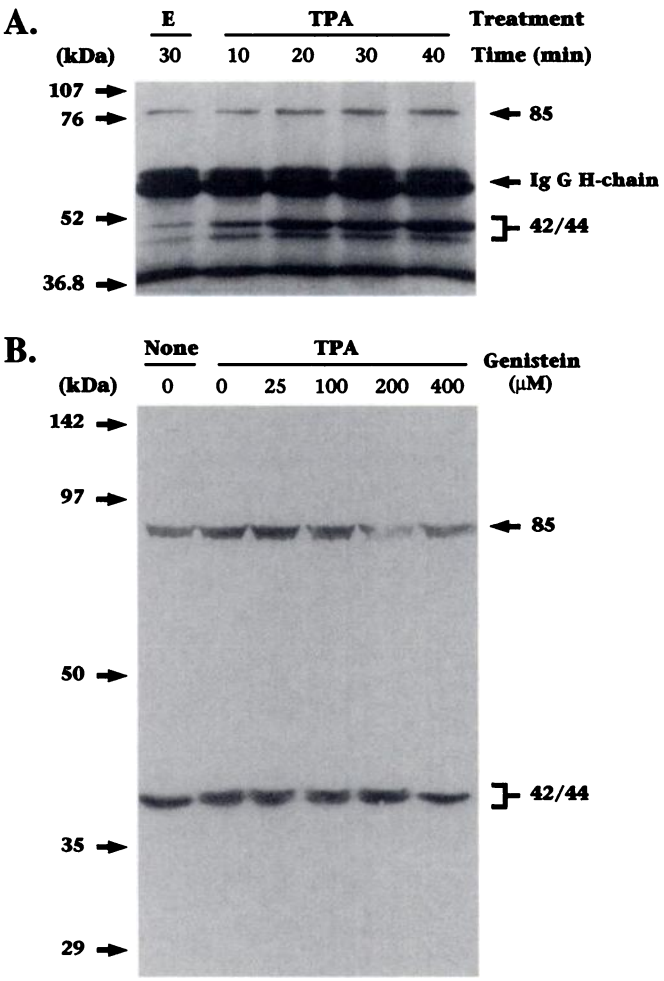

Fig. 4. Effect of genistein on TPA-induced MAPK tyrosine phosphorylation in CV-1 cells. A, Time course of TPA-induced MAPK tyrosine phosphorylation. CV-1 cells were cultured as described in legend for Fig. 1A. After treatment of CV-1 cells with 100 nM TPA or ethanol for the indicated time, cells were lysed in lysis buffer A. Immunoprecipitation of tyrosine phosphorylated proteins was performed with the antiphosphotyrosine antibody PY20. After three washes with radioimmunoprecipitation assay buffer, the immunocomplexes were dissolved in 1× SDS sample buffer and fractionated on a 10% SDS-polyacrylamide gel. The MAPK proteins were detected with anti-pan MAPK antibody coupled with ECL detection. B, Inhibitory effect of genistein on TPA-induced MAPK tyrosine phosphorylation. After pretreatment with the indicated concentration of genistein for 2 hr, cells were treated with 100 nM TPA for 30 min. CV-1 cells were lysed directly with 1× SDS sample buffer. Cell lysates were fractionated on a 10% SDS-polyacrylamide gel. The MAPK proteins were detected with anti-pan MAPK antibody coupled with ECL detection.

mRNA may also play a key role in TPA-induced ODC activity (7, 54), we determined whether genistein inhibits the phosphorylation of p90RSK or p70S6K. We first determined the effects of TPA on the phosphorylation of p90RSK and p70S6K. CV-1 cells were harvested at the indicated time (15, 30, 45, 60, and 90 min) after TPA treatment. The phosphorylation of p90RSK and p70S6K was analyzed by Western blot analysis using the anti-p90RSK or anti-p70S6K antibody. TPA treatment induced the phosphorylation of these two protein kinases with distinct kinetics (Fig. 5). p90RSK was fully phosphorylated at 15 min, whereas the p70S6K was fully phosphorylated at 30 min after TPA treatment.

We further analyzed whether the TPA-induced phosphorylations of p90RSK and p70S6K could be inhibited by a low concentration (25 μM) of genistein. Genistein, ≤200 μM, did not affect the TPA-induced phosphorylation of p90RSK (Fig.

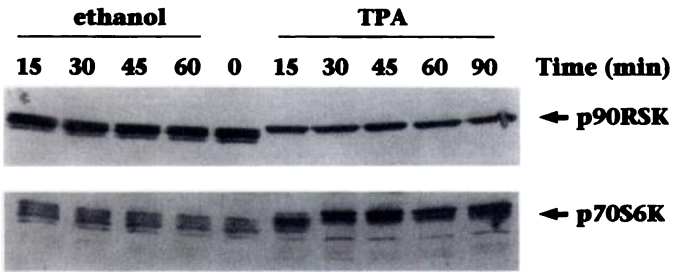

Fig. 5. TPA-induced phosphorylation of p90RSK and p70S6K. CV-1 cells were cultured as described in Fig. 1. After treatment with 100 nM TPA or ethanol for the indicated time, cells were lysed in lysis buffer B. Equal amounts of protein (100 μg) were fractionated on a 7.5% SDS-polyacrylamide gel. The expression of p90RSK, and p70S6K were detected with specific antibodies coupled with ECL detection.

6A). The band intensity of the phosphorylated form of p90RSK did not significantly change. The phosphorylated form of p70S6K with the slowest mobility seemed to be the major target of genistein. We consistently observed a 50% decrease in the TPA-induced phosphorylation of p70S6K after treatment with genistein (Fig. 6, B, *top*, and C). Essentially similar results were observed when the effect of genistein on TPA-induced phosphorylation of p70S6K was determined by immunoprecipitation using anti-p70S6K antibody (Fig. 6B, *bottom*). A decrease in the p70S6K activity by genistein was also observed with immunoprecipitation followed by an immunocomplex kinase assay (data not shown). These results indicate that p70S6K may be another molecular target of genistein and may be involved in the regulation of TPA-induced ODC activity.

Discussion

We used the CV-1 cell model to study the effects of genistein on TPA-induced ODC activity. This cell line has been shown to respond to TPA by causing induction of ODC activity (7). Furthermore, the fact that the expression of ODC activity was not proportional to the expression of ODC mRNA suggests multiple regulation of TPA-induced ODC activity in this cell line (7). We show that inhibition of TPA-induced ODC activity by genistein may involve effects on both the transcriptional and translational levels of ODC.

At a low concentration (25 μM), genistein inhibits the TPA-induced ODC activity without affecting the expression of ODC mRNA and MAPK tyrosine phosphorylation. At this dose, we also observed an inhibition of the activation and phosphorylation of p70S6K but not of p90RSK. Only the phosphorylated form of p70S6K with the slowest mobility has been shown to coelute with the active enzyme on a MonoQ column (55). Genistein seems to target p70S6K by inhibiting hyperphosphorylation (Fig. 6, B and C). These findings are in accord with the previous report that showed the inhibitory effect of genistein on S6 kinase activity in NIH 3T3 cells (43). We present findings that p70S6K is the molecular target of genistein among the S6 kinase species (Fig. 6). It has been shown that p70S6K may be a physiologically relevant kinase. This kinase phosphorylates S6 protein, which controls the translation of a subset of proteins (56). A correlation between the inhibition of TPA-induced ODC activity and TPA-induced p70S6K activation indicates that genistein may inhibit ODC activity by inhibiting the translation of ODC mRNA. This correlation is specific to ODC expression because the TPA-induced SAMD activity is not altered by 25 μM genistein (Fig. 1C). By using rapamycin as a specific inhibitor of p70S6K, Jefferies *et al.* (26) reported that p70S6K may act on the translation of a subset of proteins with pyrimidine tract mRNA. These proteins include transcripts for ribosomal protein S6, elongation factors of protein synthesis, and proteins of as-yet-unknown function (26). Because we were unable to observe a consensus pyrimidine tract on the ODC mRNA sequence, p70S6K may indirectly regulate ODC gene translation by interacting with certain translational factors.

The eIF-4F is an ATP-independent cap binding protein that possesses an ATP-dependent mRNA unwinding activity. It is composed of three nonidentical polypeptides of 25, 46, and 220 kDa (57, 58). The p25 polypeptide is the mRNA cap binding subunit, which can also be recovered as a free form, termed eIF-4E (59, 60). It is noteworthy that eIF-4F complex is phosphorylated by multifunctional S6 kinase purified from liver, which results in a stimulation of protein synthesis at initiation (61). Overexpression of eIF-4E has been shown to increase the expression of ODC activity but not SAMD activity in NIH 3T3 cells (62). These findings support our hypothesis that p70S6K plays a role in the modulation of the translational rate of ODC mRNA. Based on these observations, we propose that p70S6K activation and subsequent interaction with the translational initiation factors, such as eIF-4E, may be involved in the control of the translational rate of ODC mRNA.

p70S6K is activated by phosphorylation at four serine residues displaying serine/threonine-proline motifs (63). Phosphorylation is induced by several mitogens, such as TPA, insulin, and EGF (64–66). However, the signaling pathway that leads to the phosphorylation of p70S6K is not well understood. The inhibition of TPA-induced phosphorylation of p70S6K by the tyrosine kinase inhibitor genistein indicates that tyrosine kinase activity may be involved in the signal transduction pathway that leads to phosphorylation and activation of p70S6K. However, there is no report of tyrosine phosphorylation of p70S6K. Therefore, the putative tyrosine kinase has to be at least one step upstream of p70S6K. This kinase would then activates a serine/threonine kinase, followed by the phosphorylation and activation of p70S6K. Alternatively, the tyrosine kinase activity may be involved in the dephosphorylation of p70S6K. This would be possible if a putative phosphatase activity that dephosphorylates the p70S6K is controlled by tyrosine kinase activity. We favor the first hypothesis because the activation of serine/threonine kinase by tyrosine kinase has been well demonstrated in the MAPK activation system and is well conserved from yeast to mammalian systems in controlling gene transcriptional regulation (32, 67). A similar strategy may be used in the cell to activate p70S6K and regulate translation.

We are aware of the evidence indicating the inhibition of protein histidine kinase and topoisomerase II by genistein (68, 69). However, in these studies, the IC_{50} values for protein histidine kinase and topoisomerase II are ~110 μM–1 mM (68, 69). Conversely, the concentration we used to observe inhibitory effect on TPA-induced ODC activity and TPA-induced phosphorylation of p70S6K was as low as 25 μM. This suggests that protein histidine kinase activity or topoisomerase activity may not be involved in the activation of p70S6K or TPA-induced ODC activity.

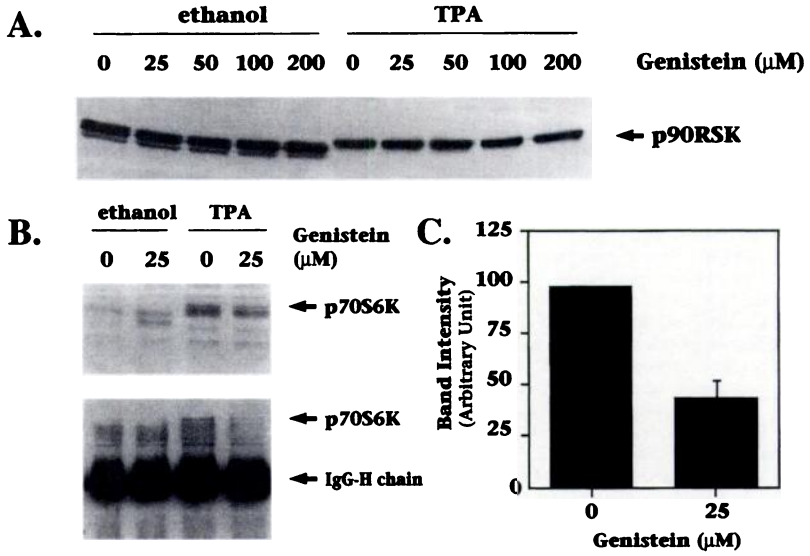

Fig. 6. The effect of genistein on TPA-induced phosphorylation of p90RSK and p70S6K. A, Effect of genistein on TPA-induced phosphorylation of p90RSK. CV-1 cells were cultured as described in Fig. 1. After pretreatment with the indicated concentration of genistein (0, 25, 50, 100, and 200 μM) for 2 hr, cells were treated with 100 nM TPA or ethanol for 45 min and lysed in lysis buffer B. Equal amounts of protein (100 μg) were fractionated on a 7.5% SDS-polyacrylamide gel. The expression of p90RSK was detected with the specific antibodies coupled with ECL detection. B, Effect of genistein on TPA-induced phosphorylation of p70S6K. After the indicated treatment, CV-1 cell extracts were prepared as described in A. Total cell extracts (100 μg, *top*) or p70S6K immunoprecipitated complex (*bottom*) were fractionated on a 7.5% SDS-polyacrylamide gel. The expression of p70S6K was detected with specific antibodies coupled with ECL detection. Similar results were obtained in two separate experiments. C, Quantification of expression levels of the phosphorylated form of p70S6K in B and additional repeated experiments were done with soft laser densitometry. Data represent the phosphorylation level of p70S6K (band with the slowest mobility) after indicated treatment relative to the DMSO/TPA-treated cells. Each value is the mean ± standard error of four experiments.

We have shown previously that TPA treatment of CV-1 cells resulted in maximal induction of ODC mRNA after 9 hr of treatment (7). Maximum activation of MAPK occurs at 30 min after TPA treatment (Fig. 4). Thus, MAPK activation precedes the induction of ODC mRNA. These results indicate a temporal relationship between MAPK activation and ODC mRNA expression. We also observed that ODC mRNA levels and MAPK tyrosine phosphorylation were both inhibited at a high concentration of genistein (i.e., 400 μM). These findings indicate the involvement of MAPK activity in TPA-induced ODC mRNA expression and suggests that the inhibitory effect of genistein on TPA-induced ODC mRNA may be mediated through the inhibition of MAPK tyrosine phosphorylation. These findings are in accord with the findings of L'allemain *et al.* (70) and Butler-Gralla and Herschman (71, 72), who reported that lack of TPA-induced MAPK activity correlated with the lack of TPA-induced ODC activity in TPA-resistant NIH 3T3 variants. The results (Table 1) of the effects of genistein on CV-1 cell viability argue against cell death as the cause of inhibition of TPA-induced ODC mRNA level and MAPK tyrosine phosphorylation by genistein. In addition, not all tyrosine-phosphorylated proteins were inhibited by genistein (Fig. 3). These results indicate the specific effect of genistein on inhibition of MAPK tyrosine phosphorylation. It is noteworthy that Winitz *et al.* (73) also reported that genistein inhibited EGF-induced but not carbachol-induced MAPK tyrosine phosphorylation, indicating the specific effect of genistein.

In summary, we report the existence of multiple mechanisms in the inhibition of TPA-induced ODC activity by genistein (Fig. 7). One of the pathways, which is sensitive to low concentration of genistein, may involve p70S6K activity and the translational control of ODC mRNA. The other pathway, which is sensitive to high concentrations of genistein, may involve MAPK activity and the transcriptional control of the ODC gene. Different tyrosine kinases may be involved in these two pathways. It seems that one of the cellular effects on cell growth by genistein may be mediated through the inhibition of ODC activity, a key enzyme in cell growth and proliferation.

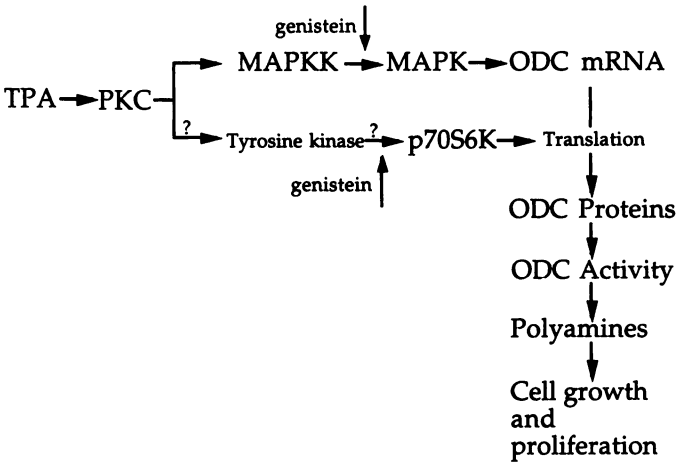

Fig. 7. Proposed mechanisms of action by genistein on TPA-induced ODC activity. *PKC*, protein kinase C; *MAPKK*, MAPK kinase.

Acknowledgments

We are grateful to Mrs. R. Simsiman for assistance in the ODC activity assay. We thank Mr. Peter J. Reddig for his helpful discussions.

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