Competitive Inhibition of Choline Phosphotransferase by Geranylgeraniol and Farnesol Inhibits Phosphatidylcholine Synthesis and Induces Apoptosis in Human Lung Adenocarcinoma A549 Cells*

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We have previously shown that, among various isoprenoids, farnesol and geranylgeraniol specifically induced actin fiber disorganization, growth inhibition, and apoptosis in human lung adenocarcinoma A549 cells (Miquel, K., Pradines, A., and Favre, G. (1996) Biochem. Biophys. Res. Commun. 225, 869–876). Here we demonstrate that isoprenoid-induced apoptosis was preceded by an arrest in G₀/G₁ phase. The isoprenoid effects were independent of protein prenylation and of mitogen-activated protein kinase activity. Moreover, geranylgeraniol and farnesol induced a rapid inhibition of phosphatidylcholine biosynthesis at the last step of the CDP-choline pathway controlled by choline phosphotransferase and not at the level of CTP:phosphocholine cytidylyltransferase, the key enzyme of the pathway. Inhibition of choline phosphotransferase was confirmed by in vitro assays on microsomal fractions, which clearly showed that the isoprenoids acted by competitive inhibition with the diacylglycerol binding. Exogenous phosphatidylcholine addition prevented all the biological effects of the isoprenoids, including actin fiber disorganization and apoptosis, suggesting that inhibition of phosphatidylcholine biosynthesis might be the primary event of the isoprenoid action. These data demonstrate the molecular mechanism of geranylgeraniol and farnesol effects and suggest that the mevalonate pathway, leading notably to prenylated proteins, might be linked to the control of cell proliferation through the regulation of phosphatidylcholine biosynthesis.

Isoprenoids are essential compounds required for cell proliferation and differentiation. Sterol and nonsterol isoprenoids are derived from a common precursor mevalonate, which is the product of 3-hydroxy-3-methylglutaryl (HMG)¹-CoA reductase (EC 1.1.1.34), the major rate-limiting enzyme in isoprenoid biosynthesis in mammalian cells (1). Cell proliferation requires at least two derivatives of mevalonate: cholesterol, necessary for cell membrane assembly, and nonsterol products involved in the mechanisms controlling initiation of DNA synthesis and cell growth (1–3) These include farnesyl- and geranylgeranyl pyrophosphate, which are required for prenylation, a post-translational maturation of diverse proteins involved in cell proliferation (4). The inhibition of protein prenylation leads to suppression of cell growth (5) to a block in cell cycle phases (6, 7) and to apoptosis (7).

The control of the isoprenoid availability is also an essential step in cell proliferation and differentiation. Complete inhibition of isoprenoid biosynthesis following addition of competitive inhibitor of HMG-CoA reductase (lovastatin or compactin) has been shown to induce growth inhibition and apoptosis (8–11). Products of mevalonate metabolism regulate proliferation of various cell lines (12). Indeed, exposure of cells to free isoprenoids such as farnesol (FOH) and geranylgeraniol (GGOH) resulted in the inhibition of cell proliferation (2, 13). FOH, GGOH, or dolichyl phosphate have also been shown to have apoptosis-inducing activity on tumor cell lines (14–17). In the human promyelocytic cell line HL60, GGOH-induced apoptosis was lately reported to be accompanied by the activation of caspase-3 protease (18). Recently, we have shown that, among various isoprenoids, GGOH and FOH specifically led to actin fiber disorganization combined with an inhibition of cell proliferation and induction of apoptosis in the human lung adenocarcinoma A549 cell line (11). In the search for a mechanism of the FOH-induced growth inhibition and apoptosis of acute leukemia cells (CEM-C1), the inhibition of phosphatidylcholine (PC) biosynthesis has been implicated (19).

PC is the most abundant phospholipid in mammalian cells, representing up to 50% of the membrane phospholipids. PC is synthesized mainly through the CDP-choline pathway regulated by the CTP:choline cytidylyltransferase (CT) (20, 21). Recent studies have implicated the PC and the CDP-choline pathway as an important component in the control of cell proliferation and cell death (22–25). Indeed, an increase of PC biosynthesis occurs when quiescent cells are triggered to enter the cell cycle (26–29). Moreover, PC is required for the normal progression through the cell cycle as shown by accumulation in the G₁ phase of fibroblasts cultured in choline-deficient medium (25). Finally, inactivation of CT in a CHO thymosensitive mutant (MT-58) (30) triggers cell death through an apoptotic process (24). Apoptosis also occurs upon inhibition of CT in cells treated with antitumor phospholipid analogues (31, 32). Although it has been reported that the CDP choline pathway for PC biosynthesis is inhibited by the free isoprenoid FOH.
Competitive Inhibition of CPT Triggers Apoptosis

in leukemia cells (33), the precise mechanisms underlying this inhibition remain to be elucidated.

In the present study we have investigated whether the inhibitory effect of FOH on the human lung adenocarcinoma A549 cell proliferation could be related to an inhibition of PC synthesis. Moreover, we have analyzed whether GGOH induced apoptosis through a similar mechanism. We demonstrate here that both GGOH and FOH are able to induce a G/G0 block and trigger apoptosis in A549 cells independently of the prenylation of proteins. We also show that GGOH, as well as FOH, induces a very rapid inhibition of PC biosynthesis at the level of choline phosphotransferase (CPT), the last step of CDP-choline pathway. Both GGOH and FOH appear to act as competitive inhibitors of CPT. These data provide the first demonstration of apoptosis induced by direct inhibition of CPT and suggest a potential coordinated regulation between the cholesterol and phospholipid biosynthesis pathways that is implicated in cell proliferation and death control.

EXPERIMENTAL PROCEDURES

Chemicals—RPMI 1640 medium, phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Life Technologies Inc. Egg yolk PC, 1,2-dioleoyl-rac-glycerol, FOH, GGOH, farnesylacetate, geraniol, choline chloride, and propidium iodide were from Sigma. [Methyl-3H]choline chloride (85 Ci/mmol), CDP-[methyl-14C]choline (62 Ci/mmol), and phosphoryl-[methyl-14C]choline (50 Ci/mmol) were supplied by Amersham Pharmacia Biotech.

Cell Culture—Human lung adenocarcinoma A549 cells (ATCC-CCL 185) were routinely grown at 37°C in RPMI 1640 medium (Life Technologies Inc.) supplemented with 5% fetal calf serum and 1% TX100, 10% glycerol, 5 mM MgCl2,1 mM EDTA, 2 mM Na3VO4,10 μM of FOH or GGOH in the absence or presence of 65 μM DG or 65 μM egg yolk PC for 48 h. Cells were fixed in 3% paraformaldehyde, PBS for 20 min, incubated in 50 mM NH4Cl, PBS for 10 min, and then permeabilized in 0.1% TX100, PBS for 3 min. Cells were stained with 5 units/ml rhodamin phalloidin (Molecular Probes) for 30 min in a humidified chamber. Cells were examined with a Zeiss microscope through a 100× oil immersion lens.

RESULTS

GGOH and FOH Induced a G/G0 Block and Apoptosis—We recently demonstrated that among diverse isoprenoids, only GGOH and FOH induce actin cytoskeleton disorganization, growth inhibition, and apoptosis (11). We have now evaluated the ability of GGOH and FOH to alter cell cycle distribution. After a 24-h attachment period, control cells displayed a typical repartition between the different phases of the cell cycle. Of total cells, 38% were in the G0/G1 phase, 38% were in the S phase, and 24% were in the G2/M phase (data not shown). As shown in Fig. 1A, after a 24-h incubation, GGOH and FOH increased the number of cells in G0/G1 up to 52 and 62%, respectively, whereas two structural analogues, geraniol and farnesylacetate, did not modify cell cycle distribution. The effect of GGOH and FOH was maximal after 24 h of treatment; an additional 24-h incubation did not significantly amplify the response (Fig. 1A). Quantification of histone-associated DNA fragments by enzyme-linked immunoassorty assay clearly indicated that treatment of A549 cells by GGOH and FOH triggered apoptosis (Fig. 1B), confirming our previous work (11). Interestingly, the G0/G1 block (Fig. 1A) as well as induction of apoptosis (Fig. 1B) were greater after cell treatment with GGOH than with FOH. By contrast, after a 48-h incubation, neither 100 μM geraniol nor farnesylacetate induced apoptosis (Fig. 1B).

Absence of Effect of GGOH and FOH on Protein Prenylation—Being structurally similar to and metabolites of farnesyl- and geranylgeranyl pyrophosphate, which are substrates for protein prenylation, GGOH and FOH could act as competitive...
inhibitors of the prenyltransferases, farnesyl- and geranylgeranyltransferase I, like other analogues such as farnesylamine (37).

To investigate whether GGOH and FOH were able to alter protein prenylation, A549 cells were treated with the isoprenoids for 48 h, and the cell lysates were immunoblotted with antibodies directed to Ha-Ras and Rap1A, two small G-proteins that are farnesylated and geranylgeranylated, respectively. In additional controls, cells were treated with FTI-277, an inhibitor of farnesyltransferase (38), or by GGTI-298, an inhibitor of geranylgeranyltransferase I (39). As expected, FTI-277 inhibited farnesylation of Ha-Ras, and GGTI-298 inhibited geranylgeranylation of Rap1A, as shown by the appearance of the upper band, which corresponds to the unprocessed form of the small G proteins (Fig. 2A). By contrast, GGOH and FOH were unable to inhibit neither Ha-Ras or Rap1A processing, as indicated by the presence of only the processed form (lower band, Fig. 2A). Moreover, GGOH and FOH did not inhibit the activity of purified farnesyltransferase determined in an in vitro assay (data not shown). These data indicated that the effects on the cell cycle of GGOH and FOH were not related to protein prenylation.

Because the prenyl group might be required for protein-membrane or protein-protein interactions (40) we cannot exclude that GGOH or FOH could inhibit such interactions. To test this hypothesis we examined whether the isoprenoids could prevent Ras membrane anchorage and interfere with the Ras-dependent signaling pathway as shown for S-farnesylthio salicylic acid (41). The activation by EGF of the Ras-dependent mitogen-activated protein kinases or ERKs was analyzed in A549 cells treated or not by GGOH or FOH. As shown in Fig. 2B, after an incubation of 24 h, GGOH and FOH inhibited neither basal nor EGF-stimulated phosphorylation of ERK1 (p44) and ERK2 (p42). These results indicated that the isoprenoids did not prevent Ras membrane localization where it can still induce its Raf-1-dependent signaling pathway. These data suggested also that the GGOH and FOH effects are independent of ERK pathway.

GGOH and FOH Inhibited PC Biosynthesis—It was previously reported that inhibition of PC biosynthesis by FOH may be the origin of apoptosis in leukemia CEM-C1 cells (19, 33). To know whether GGOH and FOH as well might act by a similar mechanism in adenocarcinoma A549 cells, the effect of these free isoprenoids on PC biosynthesis has been examined. As shown in Fig. 3A, when GGOH or FOH were added simultaneously with [3H]choline, a strong and rapid inhibition of choline incorporation into PC was observed. By contrast, the rate of PC biosynthesis in the presence of two analogues, farnesylacetate or geraniol, was comparable with the control. After 3 h of incubation, inhibition of choline incorporation into PC was almost maximal at 60 μM GGOH or FOH (80 μM) for 24 h and with epidermal growth factor (EGF) (20 ng/ml) for the last 10 min of the incubation. Cell lysates were analyzed by Western blotting with anti-Ha-Ras or anti-Rap1A antibodies as described under “Experimental Procedures.” The processed form (p) of proteins migrates faster in SDS-polyacrylamide gel electrophoresis than the unprocessed form (u). B, cells were treated or not with FOH (80 μM) or GGOH (80 μM) for 24 h and with epidermal growth factor (EGF) (20 ng/ml) for the last 10 min of the incubation. Cell lysates were analyzed by Western blotting with anti active™-ERKs or with anti-ERK antibodies. cont, control.
confirming that CPT is the site of inhibition in GGOH- and FOH-treated cells.

**GGOH and FOH Inhibited CPT Activity but Activated CT**—
CPT and CT activities were measured in subcellular fractions (cytosol or microsomes) of cells treated with GGOH or FOH for 3 h. As shown in Table I, CPT activity in the microsomal fraction was decreased in GGOH- or FOH-treated samples, confirming the radiolabel accumulation into the CDP-choline pool previously demonstrated (Fig. 4). CT activity was measured both in the microsomal and cytosolic fractions since this dual subcellular localization is a general feature of this enzyme (20, 21). Interestingly, GGOH and FOH induced a decrease of CT activity in the cytosol and an increase of CT activity in microsomes.

**Prevention of the Cellular Effects of GGOH and FOH by Phosphatidylcholine or Dioleoylglycerol Addition**—The above results suggested that the effect of GGOH and FOH on PC biosynthesis correlated with the accumulation of cells in G₀/G₁. The above results suggested that the effect of GGOH and FOH on PC biosynthesis correlated with the accumulation of cells in G₀/G₁.
leading to apoptosis. To ascertain that the inhibition of PC synthesis by the isoprenoids initiated programmed cell death, A549 cells were incubated with GGOH or FOH in the presence of exogenous DG. The presence of 65 μM DGPC was able to entirely prevent induction of G0/G1 block (Fig. 6A) and apoptosis (Fig. 6B) by both isoprenoids.

Surprisingly, the same result was obtained with diacylglycerol, the lipid substrate of the inhibited enzyme CPT. Indeed, the presence of 65 μM DG in the culture medium was able to entirely prevent induction of G0/G1 block (Fig. 6A) and apoptosis (Fig. 6B) by the isoprenoids.

To test whether PC or DG prevention of isoprenoids effects was not the result of a trapping of GGOH and FOH by the lipid vesicles added to the incubation medium, we have tried to prevent another cellular effect of GGOH by addition of PC or DG. Indeed, we have previously shown that a low concentration of GGOH (20 μM) was able to prevent the G0/G1 block induced by compactin, an inhibitor of isoprenoid synthesis (Ref. 7 and Fig. 6C). This GGOH prevention effect could be still observed whether PC or DG were added simultaneously or not, indicating that the lipids did not interact with GGOH, which could still reach its intracellular targets (Fig. 6C).

Since we have recently demonstrated that GGOH and FOH induced actin cytoskeleton disorganization in A549 cells (11), we investigated whether the addition of DG or PC prevented the effects of GGOH and FOH on the actin cytoskeleton. Examination of the actin cytoskeleton with rhodamine-phalloidin showed that untreated A549 cells contained numerous and thick actin cables that traverse the entire cell and extend to the plasma membrane (Fig. 7). The addition of exogenous DG or PC did not modify the actin cytoskeleton organization. Cell treatment with 80 μM GGOH or FOH led to a complete disappearance of actin fibers, but the addition of 65 μM DG or PC was very potent in preventing the disorganization of actin cytoskeleton induced by GGOH or FOH (Fig. 7).

GGOH and FOH Altered CPT Activity by a Competitive Inhibition with DG—The mechanism of inhibition of CPT by isoprenoids was next investigated in vitro. Increasing amount of GGOH or FOH was added directly to the microsomal fractions of untreated cells, and the CPT assay was performed. CPT inhibition was detected at 10 μM isoprenoids and reached a maximum at 60 μM (Fig. 8A). Experiments with variable amounts of DG indicated that inhibition of CPT activity by GGOH or FOH was competitive with respect to DG substrate (Fig. 8B). The isoprenoids did not modify Vmax values but significantly increased Km values for DG (from 0.042 mM up to 0.127 mM), as shown in Table II. These results confirmed that GGOH and FOH were inhibitors of CPT and suggested strongly that they acted by interfering with DG binding.

**DISCUSSION**

Our results lead to the novel conclusions that GGOH and FOH behave as inhibitors of PC biosynthesis, inducing an inhibition of cell proliferation and an arrest in G0/G1 phase leading to apoptosis. Whereas the accumulation of the cells in the G0/G1 phase was almost maximal after 24 h, the highest percentage of apoptotic cells could be seen only after 72 h. This result suggested that GGOH and FOH might induce some intracellular events causing an G0/G1 arrest leading subsequently to programmed cell death. This is in agreement with a number of data indicating that entry in apoptosis most often follows a block in G1 phase (42).

The effect of GGOH and FOH appeared specific because treatment with the same concentration of two related isoprenoids, geraniol or farnesylacetate, did not produce any alteration in the cell cycle distribution nor trigger apoptosis. These compounds are able to penetrate into the cells as indicated by their capacity to inhibit HMG-CoA reductase activity (11). In contrast, Meigs et al. (43) described an inhibition of DNA replication in Chinese hamster ovary and HeLa cells following treatment with 113 μM farnesylacetate. However in that cellular model, farnesylacetate displayed cytotoxic effects but did not cause cell death. Farnesol and analogues such as farnesylacetate and ethyl farnesyl ether have also been shown to lower the steady-state level of HMG-CoA reductase, the key regulatory enzyme of isoprenoid synthesis from mevalonate (11, 44, 45).
Thus the possibility that GGOH and FOH induced cell apoptosis by decreasing mevalonate production could have been considered. However, we had previously demonstrated that GGOH and FOH were not acting on cell growth throughout a metabolic block of the isoprenoid biosynthesis (11). Indeed, although GGOH and FOH lower HMG-CoA reductase steady-state level, this is not sufficient to affect cell proliferation, as is evidenced by the fact that inhibiting the enzyme with cholesterol had no effect on A549 cell proliferation.

Being structurally similar to, and derivatives of farnesyl- and geranylgeranyl-pyrophosphate, which are substrates for protein prenylation, GGOH and FOH could act as competitive inhibitors of prenyltransferase activity like other analogues such as farnesylamine (37). However GGOH and FOH did not inhibit the prenylation of Ha-Ras (farnesylated protein) or Rap1A (geranylgeranylated protein) (Fig. 2). Moreover GGOH and FOH did not interfere with the farnesyltransferase activity in vitro (data not shown) as previously observed for farnesylacetate (43). We were able to exclude also that GGOH or FOH inhibit prenylated protein membrane or prenylated protein-protein interactions. Indeed GGOH and FOH did not inhibit Ras-dependent ERK activation at the opposite of S-farnesylthiosalicylic acid, which without affecting prenylation, inhibits Ras-dependent ERK activation by prevention of Ras membrane localization (41).

Since prenylation of proteins was not related to GGOH or FOH effects on A549 cells, we focused on the metabolism of PC, already described to be a primary target for the FOH effect in leukemic cells (33). Interestingly we have previously demonstrated a strong link between PC biosynthesis and the control of cell proliferation and survival (24, 25). Here we show that FOH was able to affect the CDP-choline pathway. Moreover, we demonstrate that GGOH displayed a similar rapid and complete inhibition of choline incorporation into PC. This effect appeared as specific as those on cell proliferation since neither geraniol nor farnesylacetate showed inhibition of PC biosynthesis. It should be noted that this inhibition was not due to the inability of the cells to take up choline since the intracellular choline pool appeared similar in control and isoprenoid-treated cells.

The accumulation of radiolabeled CDP-choline and the decrease in PC labeling in the presence of GGOH or FOH indicated that the isoprenoids inhibited the CPT, the last step in the Kennedy pathway. This result could be surprising since the CPT is not assumed to be the regulatory enzyme of this pathway. Indeed regulation of the CDP-choline pathway occurs through both of the first two steps of this pathway, choline kinase and CT. The former appears to be correlated with some cases of mitogenesis (21), but the latter was considered as the almost-unique candidate for the regulation of PC biosynthesis (20, 21). Interestingly, we found that inhibition of the CDP-choline pathway correlated with the in vitro inhibition of CPT activity by contrast to FOH-treated leukemic cells for which CPT activity was unchanged, in apparent contradiction to the inhibition of choline incorporation into PC (19). In addition we observed an apparent translocation of CT with a decreased activity in cytosol and an increase in particulate fraction. Both inhibition of CPT and activation of CT might account for the significant accumulation of CDP-choline shown in Figs. 4 and 5. Moreover, the most compelling evidence that these isoprenoids directly inhibit CPT activity is the competitive inhibition they display toward DG (Fig. 8B). Unfortunately, the unavailability of purified or cloned CPT prevents a thorough analysis of the exact mechanism of interaction. It could not be excluded that GGOH and FOH inhibited the other cellular enzymes that require diacylglycerol, such as PKC, as it has been demon-
strated that FOH could inhibit PKC activity in HeLa cells (46). Similarly, activation of PKC was shown to prevent induction of apoptosis by GGOH (47), although in contrast, it was also reported that down-regulation of PKC suppresses induction of apoptosis (48). The effect of GGOH and FOH on PKC in our cellular model is under current investigation. However the prevention of apoptosis in the presence of PC suggested that the inhibition of the CDP-choline pathway was the primary event in the GGOH and FOH effects.

Although it is clear that cell cycle controls phospholipid biosynthesis, it was less evident whether the cell cycle and cell survival are in turn governed by PC synthesis. Several reports suggest that PC turnover is likely to be an important determinant of a normal cell cycle progression through the G1 phase (24, 25, 31, 33). PC was shown to reverse the effect of the ether lipid ET-18-OCH3 on apoptosis but not on the G0/G1 block (31). In fact, the ability of added PC to overcome the GGOH- and FOH-induced G0/G1 block (Fig. 6A) as well as apoptosis (Fig. 6B) strengthens our hypothesis. Interestingly, GGOH and FOH were also able to disrupt actin fibers, an effect reversed by adding PC or DG. Whether actin fiber disorganization is a result of induction of apoptosis or a direct consequence of the inhibition of PC biosynthesis is under investigation.

The role of the isoprenoids GGOH and FOH on cell proliferation is of paramount importance since GGOH and FOH are not only pharmacological agents but may also be produced by eucaryotic cells (49). For example, FOH metabolized from farnesyl-pyrophosphate is a terminal product secreted into the extracellular medium by Chinese hamster ovary cells (50, 51). Moreover it was recently demonstrated that the pyrophosphatase implicated in this metabolism is regulated by flow conditions in the isoprenoid pathway (52). The physiological role of FOH or GGOH is not yet determined, but the existence of the orphan nuclear receptors FXR (53) and LXRα (54) possessing transcriptional activity regulated by GGOH and FOH was recently published. Our data suggesting that the mevalonate pathway leading to cholesterol or prenylated proteins is potentially linked to the control of cell proliferation and survival through the regulation of the biosynthesis of the major phospholipid could bring new insights for a physiological role for GGOH or FOH.

TABLE II
Effect of addition of GGOH or FOH on CPT activity

The microsomal fraction of A549 cells was prepared as described under “Experimental Procedures,” and CPT activity was measured in the absence or presence of increasing amounts of GGOH or FOH. The $K_m$ and $V_{max}$ values were determined by analysis of the double-reciprocal plots shown in Fig. 8.

| Concentration (mM) | $V_{max}$ (nmol/min/mg) | $K_m$ (mM) |
|-------------------|--------------------------|------------|
| Control           | 0.230                    | 0.042      |
| Farnesol 3        | 0.227                    | 0.050      |
| 10                | 0.233                    | 0.065      |
| 20                | 0.231                    | 0.097      |
| 30                | 0.224                    | 0.095      |
| 60                | 0.206                    | 0.127      |
| Geranylgeraniol 3 | 0.248                    | 0.067      |
| 10                | 0.264                    | 0.117      |
| 20                | 0.237                    | 0.091      |
| 30                | 0.230                    | 0.109      |
| 60                | 0.224                    | 0.137      |

Fig. 7. Prevention of isoprenoid-induced actin cytoskeleton disorganization by DG and PC addition. Cells were treated by 80 μM FOH or GGOH in the absence or presence of 65 μM DG or PC for 48 h. After fixation and permeabilization of cells, actin filaments were stained with rhodamine-phalloidin as described under “Experimental Procedures.” cont, control.

Fig. 8. In vitro inhibition of CPT activity by GGOH and FOH. The microsomal fraction of untreated A549 cells was prepared as described under “Experimental Procedures.” A, concentration inhibition of CPT activity by isoprenoids. CPT activity was measured in the presence of increasing amounts of FOH (•) or GGOH (■) added to the assay. B, kinetic analysis of CPT inhibition by GGOH and FOH. CPT was measured with increasing amounts of the substrate DG and in the absence (○) or presence of increasing amounts of FOH (●) or GGOH (■). The data are presented as double-reciprocal plots and are the means of duplicates and represent of two independent experiments.
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