Uncoupling Farnesol-induced Apoptosis from Its Inhibition of Phosphatidylcholine Synthesis*

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Genetic inactivation of the synthesis of phosphatidylcholine, the most abundant membrane lipid in eukaryotic cells, induces apoptosis. Administration of farnesol, a catabolite within the isoprenoid/cholesterol pathway, also induces apoptosis. The mechanism by which farnesol induces apoptosis is currently believed to be by direct competitive inhibition with diacylglycerol for cholinephosphotransferase, the final step in the phosphatidylcholine biosynthetic pathway. Our recent isolation of the first mammalian cholinephosphotransferase cDNA has enabled us to more precisely assess how farnesol affects phosphatidylcholine synthesis and the induction of apoptosis. Induced over-expression of cholinephosphotransferase in Chinese hamster ovary cells prevented the block in phosphatidylcholine biosynthesis associated with exposure to farnesol. However, induced over-expression of cholinephosphotransferase was not sufficient for the prevention of farnesol-induced apoptosis. In addition, exogenous administration of diacylglycerol prevented farnesol-induced apoptosis but did not relieve the farnesol-induced block in phosphatidylcholine synthesis. We also developed an in vitro lipid mixed micelle cholinephosphotransferase enzyme assay, as opposed to the delivery of the diacylglycerol substrate in a detergent emulsion, and demonstrated that there was no direct inhibition of cholinephosphotransferase by farnesol or its phosphorylated metabolites. The execution of apoptosis by farnesol appears to be a separate and distinct event from farnesol-induced inhibition of phosphatidylcholine biosynthesis and instead likely occurs through a diacylglycerol-mediated process that is downstream of phosphatidylcholine synthesis.

Apoptosis, or programmed cell death, is a means by which organisms rid themselves of unwanted cells without the induction of an inflammatory response (1, 2). Apoptosis occurs naturally during the development process and for the removal of damaged cells or normal cells that have reached the end of their life span. Misregulation of the apoptotic process is believed to be a major means by which cells can become cancerous (3, 4), and conversely many chemotherapeutic drugs for the treatment of cancer do so by preferential induction of apoptosis in cancer cells (5, 6). In a recent study, 31P NMR spectroscopy was used to study alterations in cellular metabolites upon treatment with a variety of apoptosis inducing drugs as a means of identifying a marker associated with the induction of apoptosis (7). All of the agents tested resulted in an increase in the level of CDP-choline suggesting an inhibition of phosphatidylcholine (PC) synthesis at the cholinephosphotransferase step (8, 9).

PC is the most abundant lipid present in eukaryotic cell membranes comprising ~50% of cellular phospholipid mass (10). PC is synthesized almost exclusively via the three step Kennedy pathway in most eukaryotic cell types (11–14). In this pathway, choline is phosphorylated by choline kinase to produce phosphocholine which is subsequently converted to CDP-choline by CTP:phosphocholine cytidylyltransferase with the final step catalyzed by cholinephosphotransferase through the transfer of phosphocholine from CDP-choline to diacylglycerol to produce an intact PC molecule. Genetic inactivation of PC synthesis in Chinese hamster ovary (CHO) cells by shifting a cell line containing a temperature-sensitive allele of CTP:phosphocholine cytidylyltransferase to the non-permissive temperature resulted in cell death by apoptosis, and this was prevented by expression of a wild type CTP:phosphocholine cytidylyltransferase cDNA (15).

One of the compounds used in the search for alterations in cellular metabolites using 31P NMR spectroscopy during the induction of apoptosis was farnesol, a catabolite of the isoprenoid/cholesterol biosynthetic pathway (16). Farnesol is especially interesting in that it has been demonstrated to preferentially induce apoptosis in several transformed cells versus untransformed cells (17, 18). Metabolic analyses using labeled choline determined that the addition of farnesol to several cell types in culture resulted in a rapid and dramatic inhibition of PC synthesis, and the metabolic block was due to inhibition at the cholinephosphotransferase step (8, 9, 19). Farnesol-induced apoptosis could be specifically rescued by exogenous PC or diacylglycerol administration, whereas the addition of other phospholipids was ineffective (8, 20). An in vitro enzymatic analysis of cholinephosphotransferase activity present in cell membranes indicated that farnesol inhibited PC synthesis by directly competing with diacylglycerol for binding to the active site of cholinephosphotransferase (9). Thus, it was concluded that farnesol directly competed with diacylglycerol for binding to the cholinephosphotransferase active site result-

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The abbreviations used are: PC, phosphatidylcholine; CHO, Chinese hamster ovary; PARP, poly(ADP-ribose) polymerase; PE, phosphatidylethanolamine; CEPT1, human choline/ethanolaminephosphotransferase.
ing in a dramatic inhibition of PC synthesis that directly led to apoptosis (9).

Our recent isolation of the first mammalian cholinephosphotransferase cDNA (21, 22) has allowed us to more precisely determine how farnesol induces apoptosis. Our major findings include the observation that over-expression of human cholinephosphotransferase in CHO cells prevented farnesol-induced inhibition of PC synthesis; however, it did not prevent farnesol-induced apoptosis. In addition, exogenous administration of diacylglycerol significantly reduced farnesol-induced apoptosis but did not rescue PC synthesis. Thus we have uncoupled the inhibition of PC synthesis by farnesol from the ability of farnesol to induce apoptosis. To further this conclusion, a mixed micelle assay was developed for the determination of cholinephosphotransferase activity, and it did not reveal significant inhibition of the enzyme by farnesol or its phosphorylated derivatives. Farnesol-induced apoptosis is not via inhibition of PC synthesis through direct competition with diacylglycerol for the cholinephosphotransferase active site, but is instead likely through a diacylglycerol-directed mechanism that is downstream of PC synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—(methyl-1-4)C]CDP-choline, (methyl-1-4)Choline, and (1,2-14)Chelatobenzene were purchased from American Radiolabeled Chemicals. Custom oligonucleotides, T4 DNA ligase, Tag polymerase, and all tissue culture products were obtained from Life Technologies, Inc. Lipids were purchased from Avanti Polar Lipids or Sigma. The pTRE2 vector, pTK-Hyg vector, and the Tet-On CHO-K1 cell line were purchased from CLONTECH. The T7 monoclonal antibody coupled to horseradish peroxidase was a product of Novagen. Poly(ADP-ribose) polymerase (PARP) antibodies were purchased from PharMingen. Secondary antibodies were from Bio-Rad. All other reagents were of the highest quality commercially available.

**Cell Line Construction**—Tet-On CHO-K1 cells were co-transfected with 10 μg of the pTRE2 vector containing a T7 epitope-tagged version of CEPT1 (21) and 1 μg of the pTK-Hyg selection vector using the calcium chloride method (23) and subsequently incubated in Dulbecco's modified Eagle's media containing 5% fetal bovine serum in a humidified atmosphere containing 5% CO2. After 24 h, media was changed and 300 μg/ml G418 (5) and 400 μg/ml hygromycin were added. The antibiotic-containing media were changed every 48 h for 3 weeks to select for stable integrants. Single colonies were isolated, and cell lines were tested for inducible expression of CEPT1 by the addition of 2 μg/ml of doxycycline for 24 h. Cellular membranes were isolated and incubated with SDS-polyacrylamide gel electrophoresis sample buffer at 37 °C for 20 min, separated on a 10% SDS-polyacrylamide gel electrophoresis gel, and transferred to polyvinylidene difluoride membranes. Blots were probed with a T7 epitope tag monoclonal antibody (1:10,000) coupled to horseradish peroxidase for subsequent detection using the ECL (Amersham Pharmacia Biotech) system. Confirmation of CEPT1-inducible expression was determined by performing cholinephosphotransferase enzyme assays on isolated cellular membrane protein preparations.

**Enzyme Assays**—CHO-K1 cells induced to overexpress CEPT1 (CHO-AAS-CEPT1) were placed on ice and washed twice with ice-cold phosphate-buffered saline, scraped into a microfuge and centrifuged at 15,000 × g for 5 min at 4 °C. The cell pellet was resuspended in 0.5 ml of 10 mM Hepes-HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, and complete protease inhibitor mixture (Roche Molecular Biochemicals) and passed of 10 mM Hepes-HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, and complete protease inhibitor mixture (Roche Molecular Biochemicals) by incubation at 4 °C for 10 min. The cell homogenate was centrifuged at 15,000 × g for 10 min at 4 °C to pellet unbroken cells and nuclei. The supernatant was centrifuged at 150,000 × g for 30 min at 4 °C to pellet microsomal membranes. Membranes were resuspended in 10 mM Hepes-HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, and complete protease inhibitor mixture (Roche Molecular Biochemicals) by incubation at 4 °C. The cell pellet was resuspended in 0.5 ml of 10 mM Hepes-HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, and complete protease inhibitor mixture (Roche Molecular Biochemicals) by incubation at 4 °C for 10 min. Assays were terminated by the addition of chloroform/methanol (2/1, v/v) and 0.9% KCl to facilitate phase separation. The lower organic phase was washed twice with an equal volume of 40% methanol (v/v), and the PC product in the organic phase was dried in scintillation vials and radiolabel was determined. The second method was a sodium cholate-mixed micelle method performed in 50 mM Tris-HCl (pH 8.0), 20 mM MgCl2, 20% glycerol, with 10 μM CDP-choline (0.1 mM, 2000 dpm/nmol) added to initiate the reaction and tubes were incubated at 37 °C for 10 min. Assays were terminated by the addition of chloroform/methanol (2/1, v/v) and 0.9% KCl to facilitate phase separation. The lower organic phase was washed twice with an equal volume of 40% methanol (v/v), and the PC product in the organic phase was dried in scintillation vials and radiolabel was determined. The second method was a sodium cholate-mixed micelle method performed in 50 mM Tris-HCl (pH 8.0), 20 mM MgCl2, 20% glycerol, with 10 μM diacylglycerol and 10 μM PC in 1% sodium cholate using 10–25 μg of membrane protein as the enzyme source. The assay mix was incubated at 25 °C for 5 min to allow for mixed micelle formation, then initiated by the addition of CDP-choline (0.4 mM, 2000 dpm/nmol) and incubated at 25 °C. The phosphotransferase activity determined by using the mixed micelle assay increased linearly for both substrates. The assay contained at least a 2-fold greater than Km concentration of each substrate. Assays were terminated, and product was isolated as described for assay method one. Some assays contained farnesol delivered in ethanol (or an ethanol only control) and were preincubated for an additional 0–30 min prior to the addition of labeled CDP-choline. Metabolic Labeling—CHO-K1 cells and CHO-K1 cell lines containing inducible T7-CEPT1 were grown to mid log-phase in Dulbecco's modified Eagle's media containing 5% fetal bovine serum and 34 μg/ml proline. Doxycycline was added at 2 μg/ml for 24 h to induce T7-tagged CEPT1 expression. [14C]Choline (0.2 μCi) or [14C]Chelatobenzene (0.2 μCi) was added simultaneously with the indicated concentration of farnesol. Subsequent to incubation with radiolabel, cells were washed twice with ice-cold phosphate-buffered saline and resuspended in 1 ml of methanol. Two ml of chloroform and 1.5 ml of water were added, tubes were vortexed, and then centrifuged at 2,500 × g for 10 min to facilitate phase separation. Choline-containing aqueous phase metabolites were separated in a solvent system consisting of CHCl3/CH3OH/0.6% NaCl/NH4OH (50/50/5, v/v), and ethanolamine-containing metabolites were separated using CH3OH/CH2OH/2% NH4OH (1/2/v). Phospholipids in the organic phase were routinely analyzed by thin layer chromatography on Whatman silica gel 60A plates using the solvent system CHCl3/CH3OH/H2O/CH3COOH (70/30/42, v/v). Radiolabeled bands were detected using a BIOSCAN System 200 imaging scanner, and appropriate bands were scraped into scintillation vials for radioactivity determination.

**Apoptosis Determinations**—Cells were washed with Tris-buffered saline and disrupted by the addition of 1% Triton X-100 in Tris-buffered saline containing 1 mM EDTA and complete protease inhibitor mixture (Roche Molecular Biochemicals) by incubation at 4 °C for 10 min. The cell homogenate was centrifuged at 15,000 × g for 10 min at 4 °C to pellet unbroken cells and nuclei. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Blots were probed with a mouse monoclonal antibody toward PARP (1:2000), which recognizes both the mature and caspase cleaved forms of the enzyme, and Tris-buffered saline containing 0.1% Tween 20 (w/v) and 5% skim milk powder. Blots were washed and then reprobed with a goat anti-mouse secondary antibody coupled to horseradish peroxidase (1:10,000) in the above buffer. Blots were washed and PARP protein was detected using the ECL (Amersham Pharmacia Biotech) system.

The externalization of phosphatidylserine was monitored by annexin V-fluorescein staining using the Annexin-V-FLUOS staining kit from Roche Molecular Biochemicals and visualized by fluorescence microscopy. Propidium iodide was used as a counterstain for nuclear DNA. The manufacturer's method was used except that twice as much annexin V conjugate was added to each sample as recommended.

**Protein and Lipid Mass Determinations**—Protein was determined by the method of Lowry et al. (24) using bovine serum albumin as standard. Phospholipid phosphorus was determined by the method of Ames and Dubin (25).

**RESULTS**

**Effect of Farnesol on PC Synthesis**—Metabolic labeling experiments have demonstrated that farnesol, a catabolite of the cholesterolar-renaloid pathway, dramatically inhibited PC synthesis in the cholinephosphotransferase active site in several cell types (8, 9, 19). Previous work from our laboratory resulted in the isolation of the first mammalian cholinephosphotransferase-encoding cDNA, human choline/ethanolaminephosphotransferase 1 (CEPT1), whose corresponding gene was expressed in similar levels in all tissues tested and that we believe is a ubiquitous cholinephosphotransferase responsible...
over-expression of T7-tagged hCEPT1.

anti-T7 horseradish peroxidase-conjugated monoclonal antibodies of 20

Fig. 1. Development of stable cell lines capable of inducible over-expression of T7-tagged hCEPT1. A, Western blot probed with anti-T7 horseradish peroxidase-conjugated monoclonal antibodies of 20 µg of CHO membrane protein isolated as described under "Experimental Procedures" after incubation for 24 h with or without 2 µg/ml doxycycline. The arrow indicates the CEPT1 band. B, in vitro cholinephosphotransferase activity of the isolated protein samples using the mixed micelle assay.

for PC synthesis in most tissues (21, 22). To further investigate the interaction of farnesol with the CDP-choline pathway for PC synthesis we established CHO cell lines capable of inducible over-expression of CEPT1. Two separate CEPT1-inducible cell lines were constructed and resulted in similar phenotypes and results for all studies presented, although for the sake of brevity only one clone is represented in the data shown. When the CEPT1 open reading frame was placed under the control of a doxycycline-inducible promoter the addition of doxycycline to the media resulted in a dramatic induction of CEPT1 protein as assessed by Western blot (Fig. 1A). An assay of cholinephosphotransferase activity present in these CHO cells 24 h after doxycycline treatment resulted in a 27-fold increase in measurable cholinephosphotransferase activity (Fig. 1B).

Induced over-expression of CEPT1 did not increase the rate of PC synthesis (Fig. 2, B and C). This is not surprising as the cholinephosphotransferase step is normally not rate-limiting for PC synthesis (10–14). The treatment of uninduced CHO-K1 cells with farnesol resulted in a dramatic inhibition of PC synthesis to levels ~30% of control at 4 h, the limit of our time course study (Fig. 2B). An analysis of radiolabel in the metabolites of the Kennedy pathway for PC synthesis indicated two main alterations; the first was a dramatic decrease in label associated with the phosphocholine fraction indicative of an up-regulation of CTP:phosphocholine cytidylyltransferase, and the second was a massive increase in radiolabel associated with the CDP-choline fraction. As PC synthesis was ultimately inhibited the metabolic block at the cholinephosphotransferase step was the over-riding factor that resulted in an inhibition of PC synthesis. This observation is consistent with other studies with respect to the inhibition of PC synthesis by farnesol (8, 9, 19). Upon induced over-expression of the CEPT1 protein we were able to completely reverse farnesol-induced inhibition of PC synthesis (Fig. 2C). An analysis of the radiolabeled Kennedy pathway metabolites revealed several interesting regulatory processes. The radiolabel associated with the phosphocholine fraction remained high even though the rate of PC synthesis had returned to normal levels indicating that the CTP:phosphocholine cytidylyltransferase step was still up-regulated, implying farnesol may irreversibly activate CTP:phosphocholine cytidylyltransferase. The CDP-choline level remained high implying that although the increased cholinephosphotransferase activity provided by induction of CEPT1 was able to successfully convert CDP-choline into PC, activation of the upstream CTP:phosphocholine cytidylyltransferase step kept the label associated with CDP-choline fraction high.

This study illustrates that although farnesol has pleiotropic effects within the Kennedy pathway for PC synthesis, farnesol-induced inhibition of PC synthesis is manifested at the level of the cholinephosphotransferase step. This study also provides the first data that describes the ability of the dual specificity CEPT1 choline/ethanolaminephosphotransferase to supply cholinephosphotransferase activity for the synthesis of PC in mammalian cells (20, 21).

Effect of CEPT1 on Farnesol-induced Apoptosis—Genetic inactivation of PC synthesis via the use of a temperature-sensitive allele of CTP:phosphocholine cytidylyltransferase, the step that is normally rate-limiting for PC synthesis, resulted in apoptosis in CHO cells (15). The addition of farnesol to several cell types also induced apoptosis and correlated with the ability of farnesol to inhibit PC synthesis at the cholinephosphotransferase step (8, 9, 19). Farnesol-induced apoptosis was partially rescued by the addition of diacylglycerol or PC, but not other lipids (8, 9, 20). In our study in CHO cells farnesol induced apoptosis, as monitored by cleavage of PARP and the externalization of phosphatidylserine at 80 µM and above (Fig. 3, A and B). This was a similar concentration to that observed for other cell types (8, 9, 19). A close examination of the phosphatidylserine externalization data (Fig. 3B) indicates that an estimated 80% of the cells have externalized their phosphatidylserine, whereas ~15% also have their nucleus stained with propidium iodide implying that these cells are either in a much later stage of apoptosis or have undergone necrosis. We are in the process of isolating this cell population using fluorescence cell sorting to determine their mode of death. The most notable observation from this set of experiments was that CHO cells induced to over-express CEPT1, a condition that we demonstrated restored PC synthesis to normal levels (Fig. 2), did not result in a rescue of farnesol-induced cell death (Fig. 3, A and B). This implies that direct inhibition of cholinephosphotransferase by farnesol may not be the mechanism by which farnesol induced apoptosis (9).

Effect of Farnesol on in Vitro Cholinephosphotransferase Activity—The ability of farnesol to induce apoptosis through the inhibition of PC synthesis was hypothesized to work via direct competitive inhibition of cholinephosphotransferase enzyme activity for diacylglycerol by farnesol (9). This was demonstrated using subcellular membranes as a source of cholinephosphotransferase enzyme and an enzyme assay that delivered the insoluble diacylglycerol and farnesol as an emulsion using either a very small amount of detergent or without the aid of detergent solubilizers (8, 9). We also observed that the greatest in vitro inhibition of cholinephosphotransferase activity involved preincubation of the cellular membranes with farnesol (8). We also observed a significant inhibition of cholinephosphotransferase activity by farnesol, to 32% control values, when the lipid substrates were delivered without the aid of solubilizing detergents (Table I). Preincubation of the assay mixture with farnesol for 30 min prior to the addition of the
radiolabeled CDP-choline substrate resulted in a cholinephosphotransferase activity that was 6% of control levels.

Because our metabolic labeling and induction of apoptosis data seemingly uncoupled farnesol-induced apoptosis from the inhibition of PC synthesis we hypothesized that under the conditions used in the previous analysis of cholinephosphotransferase activity farnesol may be progressively altering the membrane structure such that the integral membrane cholinephosphotransferase protein was becoming inactivated (26). To test this possibility and to further characterize the ability of farnesol to inhibit cholinephosphotransferase activity we developed a mixed micelle cholinephosphotransferase enzyme assay. The mixed micelle method results in a uniform delivery of lipid-soluble substrates and activators/inhibitors and eliminates the ability of the lipids to alter the bulk membrane environment (27). In the studies that had delivered lipids as emulsions or without the aid of detergents and had demonstrated cholinephosphotransferase inhibition with farnesol also did not result in an inhibition of cholinephosphotransferase activity. To ensure the mixed micelle assay condition used is an environment compatible with accurately measuring cholinephosphotransferase activity we tested if a previously identified inhibitor of cholinephosphotransferase, chelerythrine, was able to inhibit cholinephosphotransferase activity. Chelerythrine at 50 \( \mu \text{M} \) inhibited cholinephosphotransferase activity to 38% control values and at 100 \( \mu \text{M} \) to 25% control values when assayed using our mixed micelle system; this was a similar level of inhibition to that observed using an emulsion-based assay (8).

In the studies that had delivered lipids as emulsions or without the aid of detergents and had demonstrated cholinephosphotransferase inhibition by farnesol the net concentration of diacylglycerol was 2 mM, and the amount of farnesol required to observe significant cholinephosphotransferase activity inhibition was 60–80 \( \mu \text{M} \). In our mixed micelle system diacylglycerol was delivered as 10 mol% lipid, which corresponds to an effective concentration of 1.2 mM, and farnesol was added at 100 \( \mu \text{M} \) or 0.8 mol%. Hence, our effective ratio of

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**Fig. 2. Effect of farnesol on PC biosynthesis.** A, the Kennedy pathway for the synthesis of PC. B, radiolabeled choline was added to uninduced CHO-K1-CEPT1 cells simultaneously with 80 \( \mu \text{M} \) farnesol (closed symbols) or an ethanol control (open symbols). PC and the Kennedy pathway metabolites were separated and associated label was quantitated as described under "Experimental Procedures." C, CHO-K1-CEPT1 cells were induced to over-express CEPT1 for 24 h with 2 \( \mu \text{g/ml} \) doxycycline and were subsequently treated with 80 \( \mu \text{M} \) farnesol (closed symbols) or an ethanol control (open symbols). PC and the Kennedy pathway metabolites were separated and associated label was quantitated as described under "Experimental Procedures."
farnesol to diacylglycerol was greater than that used previously to demonstrate enzyme activity inhibition. We predict that in vitro, farnesol likely does not directly inhibit cholinephosphotransferase activity but instead can compromise the structure of the membrane used as enzyme source and progressively inactivate the enzyme.

**Effect of Diacylglycerol on Farnesol-induced Apoptosis**—Diacylglycerol is one of the substrates for cholinephosphotransferase, and administration of diacylglycerol to cells in culture was found to partially prevent farnesol-induced apoptosis (9, 20). This was one of the observations that led to the hypothesis that farnesol induced apoptosis by direct inhibition of cholinephosphotransferase activity and subsequent limitation of PC synthesis. Our results demonstrate that annexin V externalization was dramatically decreased upon addition of di18:1 diacylglycerol to farnesol-treated cells indicating protection versus apoptosis by diacylglycerol (Fig. 4). A close inspection of the results indicated that there was only a small decrease in annexin V in cells whose nucleus was stained with propidium iodide suggesting that the subset of cells stained positive by both annexin V and propidium iodide may be undergoing apoptosis as opposed to necrosis, and diacylglycerol can only rescue the apoptotic cells. Definitive evidence will require isolation of this specific population of cells and an analysis of their mode of death.

We also observed the effect of exogenous administration of diacylglycerol on farnesol-induced inhibition of PC synthesis. The addition of diacylglycerol was unable to restore PC synthesis to control levels as monitored by the incorporation of radiolabeled ethanolamine into PC and corresponding upstream metabolites of the Kennedy pathway (Fig. 5). Thus, diacylglycerol rescue of farnesol-mediated apoptosis is not via restoration of de novo PC synthesis.

**Farnesol Inhibition of Phosphatidylethanolamine Synthesis**—To test if farnesol inhibition of PC synthesis was specific to the Kennedy pathway for the synthesis of PC, we tested whether farnesol could also inhibit the analogous pathway for the synthesis of PE (28). Treatment of CHO cells with farnesol inhibited the synthesis of PE as monitored metabolically by the addition of radiolabeled ethanolamine to the medium of cells concomitant with farnesol treatment (Fig. 6). Analysis of the metabolites within the CDP-ethanolamine pathway indicated increases in label associated with ethanolamine and phosphoethanolamine, consistent with inhibition at the CTP:phosphoethanolamine cytididylyltransferase step of this pathway. This was in contrast to that observed for the Kennedy pathway for the synthesis of PC, which can synthesize both PC from CDP-choline and PE from CDP-ethanolamine in vitro and in vivo and is the only ethanolaminephosphotransferase present in mammalian cells (21, 22), was unable to rescue farnesol-induced inhibition of PE synthesis (Fig. 6B). Thus, farnesol-induced inhibition of phospholipid synthesis is pleiotropic and not limited to either the inhibition of PC synthesis or the final phosphotransferase reaction of the Kennedy pathways.

**TABLE I**

*In vitro inhibition of cholinephosphotransferase activity by farnesol without detergent solubilizers*

| Preincubation time (60 min, farnesol) | Cholinephosphotransferase activity % control |
|--------------------------------------|-------------------------------------------|
| 0                                   | 32                                        |
| 30                                  | 6                                         |

**TABLE II**

*Effect of farnesol and its analogues and metabolites on cholinephosphotransferase activity using a mixed micelle enzyme assay*

| Compound                      | Cholinephosphotransferase activity % control |
|-------------------------------|-------------------------------------------|
| 0.8 mol%–100 μM              |                                            |
| farnesol                      | 90                                        |
| farnesol (30 min preincubation)| 87                                        |
| farnesol-PP                   | 107                                       |
| geranyl-P                     | 108                                       |
| geranylgeraniol               | 97                                        |
| geranylgeraniol-PP            | 106                                       |
| chelerythrine (50 μM)         | 38                                        |
| chelerythrine (100 μM)        | 25                                        |
DISCUSSION

Farnesol is a catabolite of the cholesterol/isoprenoid biosynthetic pathway whose administration preferentially induces apoptosis in transformed versus untransformed cells (16–18). The intracellular phosphorylation of farnesol results in the synthesis of farnesol pyrophosphate, which allows for the incorporation of farnesol directly into proteins most notably small G proteins including Ras (29, 30). This protein prenylation event is an essential process for oncogenic Ras to affect cellular transformation, and the farnesyl:protein transferase step is the target of several new anticancer drugs (31, 32). The ability of farnesol administration to alter the prenylation of the small G proteins Ras and Rap1A was tested and neither the prenylation event nor the ability of the G proteins to associate with the membrane was altered (9). Thus, it appears that farnesol administration preferentially kills transformed cells by a mechanism independent of protein prenylation events.

The addition of farnesol and several other compounds that induce apoptosis resulted in the preferential accumulation of the metabolite CDP-choline as monitored by NMR spectroscopy (7). CDP-choline is used in conjunction with diacylglycerol to synthesize PC by the enzyme cholinephosphotransferase. As genetic inactivation of PC synthesis in CHO cells resulted in apoptosis (15), it was postulated that farnesol induced apoptosis through its repression of PC synthesis by direct inhibition of cholinephosphotransferase. Evidence gathered in support of this hypothesis has been substantial and includes the observations that (i) metabolic labeling of cells in culture resulted in a dramatic decrease in PC labeling with a corresponding increase in CDP-choline labeling (8, 9, 19), (ii) farnesol-induced apoptosis could be prevented by the addition of diacylglycerol or PC but not other lipids to the media of cells (8, 9, 20), and (iii) farnesol inhibited cholinephosphotransferase enzyme activity in vitro (8, 9).

To further examine the precise interaction between farnesol-induced apoptosis and the inhibition of PC synthesis at the cholinephosphotransferase step we constructed CHO cell lines capable of inducible over-expression of CEPT1, a dual specificity human choline/ethanolaminephosphotransferase capable of synthesizing PC and PE in vitro and in vivo (21, 22). Over-expression of CEPT1 prevented farnesol-induced inhibition of PC synthesis but did not prevent farnesol-induced apoptosis. This was the first demonstration that CEPT1 could synthesize PC in mammalian cells and also implied that inhibition of de novo PC synthesis may not be the mechanism by which farnesol causes apoptosis. We demonstrated that the addition of
diacylglycerol, one of the substrates of the cholinephosphotransferase enzyme, prevented farnesol-induced apoptosis (8, 9, 20), but most notably exogenous diacylglycerol administration did not reconstitute PC synthesis. The most likely explanation for the inability of diacylglycerol to restore PC synthesis, yet prevent farnesol-induced apoptosis, is that farnesol causes apoptosis by inhibiting a diacylglycerol-mediated process that is produced at an intracellular site distinct from the diacylglycerol pool used for PC synthesis.

To further study the mechanism by which farnesol affected PC synthesis we devised a mixed micelle method for the determination of CEPT1 cholinephosphotransferase enzyme activity (27). This was in contrast to the assays used previously to demonstrate the inhibition of cholinephosphotransferase activity by farnesol, which delivered lipids as an emulsion or did not include lipid-solubilizing detergents in the assay mixture (8, 9). The mixed micelle method solubilizes the membrane used as enzyme source such that the addition of exogenous lipid substrates or activators/inhibitors does not alter the physical parameters of the micelle containing the cholinephosphotransferase enzyme (27). This is not the case for the assay in which farnesol was delivered as in emulsion using a small amount of detergent or without the aid of detergents (8, 9). Using the mixed micelle assay neither farnesol nor its phosphorylated derivatives inhibited CEPT1 cholinephosphotransferase activity. We conclude from this study that farnesol likely does not directly inhibit cholinephosphotransferase activity in vitro but instead alters the physical properties of the membrane such that when farnesol is delivered in the absence of excess detergent cholinephosphotransferase is progressively inactivated. However, the issue of precisely how farnesol inhibits cholinephosphotransferase remains to be resolved. Acidification of the cytosol is associated with most forms of apoptosis and Anthony et al. (8) demonstrated that farnesol rapidly acidified the cytosol in a time dependent manner consistent with inactivation of cholinephosphotransferase activity, especially because the active site of cholinephosphotransferase is known to face the cytoplasm. However, our over-expression of CEPT1 rescued PC synthesis when cells were challenged with farnesol, and acidification should inactivate an enzyme regardless of its expression level. The same explanation could be used to discount the possibility that farnesol physically perturbs the biological membrane resulting in an inhibition of cholinephosphotransferase activity. However, there are few other known effectors of
cholinephosphotransferase activity that could be overcome by an increase in CEPT1 expression. One of these is a decrease in cellular diacylglycerol mass, which has been demonstrated to result in the cholinephosphotransferase step becoming rate-limiting for PC synthesis (33, 34), and the other is a strong in vitro inhibition of cholinephosphotransferase by Ca$^{2+}$ (35). Neither of these parameters has been measured subsequent to farnesol addition, but both are dependent on a molar amount to alter cholinephosphotransferase activity and could be overcome by increased expression of CEPT1.

The ability of diacylglycerol and PC but not other lipids to prevent farnesol-induced apoptosis (8, 9, 20), together with the data presented in our study, leads one to speculate that farnesol or one of its metabolites prevents a diacylglycerol-mediated event that is derived from PC and required for cell survival. If true, a farnesol-mediated process could either interfere with the generation of diacylglycerol produced from PC and/or affect a diacylglycerol target molecule that produces a cell survival signal. Indeed, the diacylglycerol regulated protein kinase Cα and protein kinase Cβ isoforms have been demonstrated to translocate from their active membrane-bound forms to their storage cytosolic forms upon farnesol administration (36, 37).

The precise pathway by which farnesol causes apoptosis needs to be determined in further detail to delineate how farnesol preferentially targets transformed cells versus those that are untransformed for cell death (17). Our study has clarified some of the mechanisms of cross-talk between various lipid metabolic pathways and has highlighted their importance in the balance between the generation of cell survival and apoptotic messages.

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