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Perillyl alcohol and genistein differentially regulate PKB/Akt and 4E-BP1 phosphorylation as well as eIF4E/eIF4G interactions in human tumor cells

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Abstract

Previously we demonstrated that secondary products of plant mevalonate metabolism called isoprenoids attenuate 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA translational efficiency and cause tumor cell death. Here we compared effects of "pure" isoprenoids (perillyl alcohol and γ-tocotrienol) and a "mixed" isoprenoid—genistein—on the PKB/Akt/mTOR pathway that controls mRNA translation and m^7GpppX eIF4F cap binding complex formation. Effects were cell- and isoprenoid-specific. Perillyl alcohol and genistein suppressed 4E-BP1(Ser65) phosphorylation in prostate tumor cell lines, DU145 and PC-3, and in Caco2 adenocarcinoma cells. Suppressive effects were similar to or greater than that observed with a PI3 kinase inhibitor or rapamycin, an mTOR inhibitor. 4E-BP1(Thr37) phosphorylation was reduced by perillyl alcohol and genistein in DU145, but not in PC-3. Conversely, perillyl alcohol but not genistein decreased 4E-BP1(Thr37) phosphorylation in Caco2. PKB/Akt activation via Ser473 phosphorylation was enhanced in DU145 by perillyl alcohol and in PC-3 by γ-tocotrienol, but was suppressed by genistein. Importantly, perillyl alcohol disrupted interactions between eIF4E and eIF4G, key components of eIF4F (m^7GpppX) cap binding complex. These results demonstrate that "pure" isoprenoids and genistein differentially impact cap-dependent translation in tumor cell lines.
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Secondary products of plant mevalonate metabolism—collectively called isoprenoids—are recognized for their cancer preventive and treatment capabilities [1]. The ~23,000 isoprenoids found in fruits and vegetables differ in size, complexity and function. Structurally, "pure" isoprenoids such as monoterpenes, diterpenes and triterpenes consist of multiples of the five-carbon isoprene unit. "Mixed" isoprenoids comprise isoflavones (genistein), prenylated coumarins, flavones and others, each with only a part of the molecule derived via the mevalonate pathway [2]. Pure isoprenoids including limonene, perillyl alcohol, γ-tocotrienol, β-ionone and farnesol initiate apoptosis and concomitantly arrest tumor cells in the G1 cell cycle phase [1,3]. Soy-based genistein likewise thwarts proliferation of diverse tumor cells in culture [4]. Moreover, pure and mixed isoprenoids suppress growth of numerous animal tumor models including leukemic cells, melanomas, pancreatic tumors and hepatomas [1,3].

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Our laboratory found that limonene, perillyl alcohol and geraniol suppress 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[2] reductase synthesis in mammalian cells by decreasing the translational efficiency of HMG-CoA reductase transcripts [5]. This was the first evidence that dietary monoterpenes specifically affect cellular gene expression at the protein synthetic level. Furthermore, mevalonate-mediated suppression of HMG-CoA reductase synthesis was reversed by over-expression of eukaryotic initiation factor 4E (eIF4E) [6], a key component in the protein kinase B (PKB)/Akt/mTOR signaling pathway that controls protein synthesis. Levels of eIF4E available for association with the m^7GpppX cap binding complex, eIF4F—a trimeric complex of eIF4E, eIF4A and eIF4G [7]—are regulated through eIF4E's affinity for its binding partner, 4E-BP1 [8,9]. Cellular stimulation with growth factors or insulin results in 4E-BP1 phosphorylation through the PKB/Akt cascade [10–12] via mTOR—mammalian target of rapamycin. mTOR mediates 4E-BP1 phosphorylation at residues Thr37 and Thr46 and facilitates phosphorylation at sites Ser65 and Thr70 [13]. Additional 4E-BP1 phosphorylation sites occur at Ser83 and Ser112 [9,14]. Phosphorylation reduces binding interactions between eIF4E and 4E-BP1 resulting in elevated eIF4E levels available for eIF4F formation [9]. Conversely, blocks to the PKB/Akt/mTOR cascade cause decreased 4E-BP1 phosphorylation and diminished eIF4E available for translation initiation [9].

To define further how dietary factors decrease protein translation, we examined effects of representative ''pure'' isoprenoids (perillyl alcohol and γ-tocotrienol) and a ''mixed'' isoprenoid—genistein—on the PKB/Akt/mTOR pathway and m^7GpppX eIF4F cap binding complex formation in human solid tumor cell lines. Protein phosphorylation changes were compared to known inhibitors of the pathway and to an HMG-CoA reductase inhibitor, lovastatin.

Materials and methods

Media, cells and reagents

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained as monolayers in a humidified atmosphere containing 5% CO_2. Human colorectal adenocarcinoma cells, Caco2, were grown in minimum essential medium (MEM) supplemented with 20% fetal bovine serum (FBS) and antibiotics. Human prostate cancer cell lines, PC-3 and DU145, were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

Perillyl alcohol (Aldrich Chemicals, St. Louis, MO) and γ-tocotrienol (provided by Dr. Huanbiao Mo, Texas Women's University) were dissolved in 100% ethanol to stock concentrations of 0.4 and 0.02 M, respectively. Lovastatin was provided by Merck Laboratories (Raritan, NJ) and was prepared as described previously [15]. Rapamycin (Cell Signaling, Beverly, MA) was prepared as described by Brunn et al. [16]. The MEK1 inhibitor PD98059 (Cell Signaling) was dissolved in methanol at a concentration of 50 mM. The PI3 kinase inhibitor LY294002 (Cell Signaling), p38 MAP kinase inhibitor SB 203580 (Sigma) and genistein (Santa Cruz Biotechnology, Santa Cruz, CA) were dissolved in DMSO at concentrations of 10, 20 and 80 mM, respectively.

eIF4E monoclonal antibody was from Transduction Labs (Lexington, KY); phospho-4E-BP1 (Ser65)- and phospho-4E-BP1 (Thr37)-specific antibodies as well as AKT-, eIF4G- and phospho-Akt (Ser437)-specific antibodies were purchased from Cell Signaling. Secondary antibodies were from Santa Cruz Biotechnology.

Cellular treatment and protein isolation

Cells were plated at 3×10^6 per 100 mm culture dish in appropriate medium and allowed to attach for 24 h before initiating treatment conditions. Lovastatin, perillyl alcohol and γ-tocotrienol were added to a final concentration of 5, 400 and 20 μM, respectively; incubations were done for an additional 16 h. Incubations with genistein, LY294002, PD98059 or rapamycin were done for 4 h at final concentrations of 40 μM, 10 μM, 50 μM and 10 nM, respectively. Following cellular treatment, medium was removed and cells washed twice with ice cold phosphate-buffered saline (PBS). The conditions of Graves et al. [17] were used to lyse cells. A 250 μl aliquot of RIPA cell lysis buffer (10 mM sodium phosphate, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and 0.01% NaN3) containing fresh protease inhibitors (50 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM aprotinin, 1 mM sodium vanadate and 75 μM sodium fluoride) was added to each plate. Cell lysate was scraped from the dish with a rubber policeman, the homogenate transferred to cold centrifuge tubes, and tubes agitated at 4 °C for 30 min. Homogenates were then centrifuged (15,000*g*) at 4 °C for 15 min to pellet insoluble material. Protein concentrations were determined by the Bradford assay according to manufacturer's suggested conditions (Pierce Biotechnology, Rockford, IL). An equal volume of 2× electrophoresis Laemmli buffer (0.5 M Tris, pH 6.8, 15% glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 0.25% 2-mercaptoethanol and 0.05% (w/v) bromophenol blue) was added to 100–200 μg of total protein for immunodetection of eIF4E or 4E-BP1 on Western blots. Samples were heated (90 °C for 4 min), loaded on a 12% polyacrylamide–SDS gel and electrophoresed for ~1 h at 200 V.

For analysis of phosphorylated Akt/PKB (Ser473), cells (5×10^6) were washed 1× with ice cold PBS and lysed directly with 1× Laemmli buffer according to the method of Klein and Fischer [18]. Lysates were sonicated four times for 5 s each on ice. Samples were heated at 95 °C and proteins resolved by SDS–PAGE as described above.

Western blot analysis of proteins

Electrophoresed gels were equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20% MeOH, pH 8.3) for 10 min with agitation. Proteins were transferred onto a pre-wet and equilibrated 0.2 μm pore size Immun-Blot® polyvinylidene fluoride (PVDF) membrane. Efficiency of transfer was checked by staining with Ponceau S solution (Sigma) for 5 min and destaining with H_2O for 5 min. Membranes were placed in blocking solution (5% w/v nonfat dry milk, 10 mM Tris, pH 7.5, 100 mM NaCl and 0.1% Tween 20) for 30 min at room temperature (RT) and then incubated with agitation in the appropriate primary antibody—mouse monoclonal for eIF4E (1:1000) or rabbit polyclonal antibodies for phospho-4E-BP1 (Ser65) (1:1000), phospho-4E-BP1 (Thr37), eIF4G (1:1000), or

[2] *Abbreviations used:* PI3 kinase, phosphatidylinositol 3-kinase; eIF, eukaryotic initiation factor; 4E-BP1, eukaryotic initiation factor 4E binding protein; PKB, protein kinase B; mTOR, mammalian target of rapamycin; m^7Gppp, 7-methyl GTP; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MEM, minimal essential medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; IRES, internal ribosomal entry site; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; RT, room temperature; EGFR, epidermal growth factor receptor.

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