Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 46 (2017) 49–56

Journal of Nutritional Biochemistry

Gamma-tocotrienol profoundly alters sphingolipids in cancer cells by inhibition of dihydroceramide desaturase and possibly activation of sphingolipid hydrolysis during prolonged treatment☆

Yumi Jang, Xiayu Rao, Qing Jiang*

Department of Nutrition Science, Purdue University, West Lafayette, IN 47907

Received 23 November 2016; received in revised form 11 March 2017; accepted 6 April 2017

Abstract

Vitamin E gamma-tocotrienol (γTE) is known to have anticancer effects, but mechanisms underlying these actions are not clear. Here using liquid chromatography tandem mass spectrometry, we show that γTE induced marked changes of sphingolipids including rapid elevation of dihydrosphingosine and dihydroceramides (dhCers) in various types of cancer cells. The elevation of dihydrosphingolipids coincided with increased cellular stress, as indicated by JNK phosphorylation, and was prior to any sign of induction of apoptosis. Chemically blocking *de novo* synthesis of sphingolipids partially counteracted γTE-induced apoptosis and autophagy. Experiments using $^{13}C_3$, ^{15}N–labeled L-serine together with enzyme assays indicate that γTE inhibited cellular dihydroceramide desaturase (DEGS) activity without affecting its protein expression or *de novo synthesis* of sphingolipids. Unlike the effect on dhCers, γTE decreased ceramides (Cers) after 8-h treatment but increased $C_{18:0}$-Cer and $C_{16:0}$-Cer after 16 and 24 h, respectively. The increase of Cers coincides with γTE-induced apoptosis and autophagy. Since γTE inhibits DEGS and decreases *de novo* Cer synthesis, elevation of Cers during prolonged γTE treatment is likely caused by sphingomeylinase-mediated hydrolysis of sphingomyelin. This idea is supported by the observation that an acid sphingomeylinase inhibitor partially reversed γTE-induced cell death. Our study demonstrates that γTE altered sphingolipid metabolism by inhibiting DEGS activity and possibly by activating SM hydrolysis during prolonged treatment in cancer cells.

Keywords: Sphingolipids; Vitamin E; Tocotrienol; Cancer; Autophagy; Apoptosis; Dihydroceramide desaturase

1. Introduction

Sphingolipids constitute essential components of the cell membrane and are important signaling molecules that regulate cell growth, differentiation, senescence and apoptosis [1–3]. The levels of endogenous sphingolpids are controlled by *de novo* synthesis pathway and dynamic metabolism involving catabolic and synthetic pathways of complex sphingolipids. Briefly, *de novo* sphingolipid synthesis begins in the endoplasmic reticulum from condensation of palmitoyl-CoA and serine by serine palmitoyltransferase (SPT) to form 3-keto-dihydrosphingosine, which is then reduced to form dihydrosphingosine (dhSph). dhSph is acylated by a family of (dihydro)ceramide synthases (CerSs) to generate dihydroceramides (dhCers) (Fig. 1A). In mammals, there are six identified CerSs, and each CerS has preference for using different lengths of fatty acyl CoAs as substrates, which produce distinct dhCers including $C_{16:0}$-, $C_{18:0}$-, $C_{20:0}$-, $C_{24:0}$-, $C_{24:1}$-dhCer and so on. Subsequently, dhCers are converted to ceramides (Cer) by dhCer desaturase (DEGS) that inserts a 4,5-*trans* double bond. In the Golgi apparatus, Cers are converted to more complex sphingolipids such as glucosyl- or galactosyl-Cers and sphingomyelin (SM) by glucosyl-Cer synthase, Cer galactosyltransferase and SM synthases (SMS), respectively. As for the degradation pathways, Cer can either be broken down by ceramidases into sphingosine (Sph) which may be salvaged into sphingolipid pathways or phosphorylated to form sphingosine-1-phosphate (S1P). In addition, Cers can be generated by breakdown of SM through the action of acid or neutral sphingomyelinases (SMases) (Fig. 1A) [2,4–6].

It is well established that Cers and S1P are important bioactive lipids that regulate cell stress, growth and survival [3,7–9]. Cers with different side chain are shown to have distinct activities, although this topic is an emerging area of research and requires further investigation [4,10–12]. DhCers, despite being traditionally thought of as an inactive precursor of Cers [1], have recently been found to be bioactive and appeared to be involved in important cellular responses including cell cycle arrest [13,14], apoptosis [15–17], autophagy [1,15,18] and oxidative stress

Abbreviations: γTE, γ-tocotrienol; LC–MS/MS, liquid chromatography tandem mass spectrometry; Cer, ceramide; dhCer, dihydroceramide; Sph, sphingosine; dhSph, dihydrosphingosine; SM, sphingomyelin; dhSM, dihydrosphingomyelin; DEGS, dhCer desaturase; C8-CPPC, C8-cyclopropenylceramide; SMS, sphingomyelin synthase; SMase, sphingomyelinase; LC3, microtubule-associated protein light chain 3; PARP, poly-ADP-ribose polymerase; HMG-CoA reductase, 3-hyroxy-3methylglutaryl Coenzyme A reductase.

☆ GRANTS AND FUNDING SOURCES: This study was in part supported by grants R21CA152588, R01AT006882 (to QJ) from National Institutes of Health. This project was also partially supported by NIH grant P30CA023168.

* Corresponding author at: Department of Nutrition Science, 700 W State Street, Stone Hall G1A, Purdue University, West Lafayette, IN 47907.

E-mail address: qjiang@purdue.edu (Q. Jiang).

http://dx.doi.org/10.1016/j.jnutbio.2017.04.003
0955-2863/

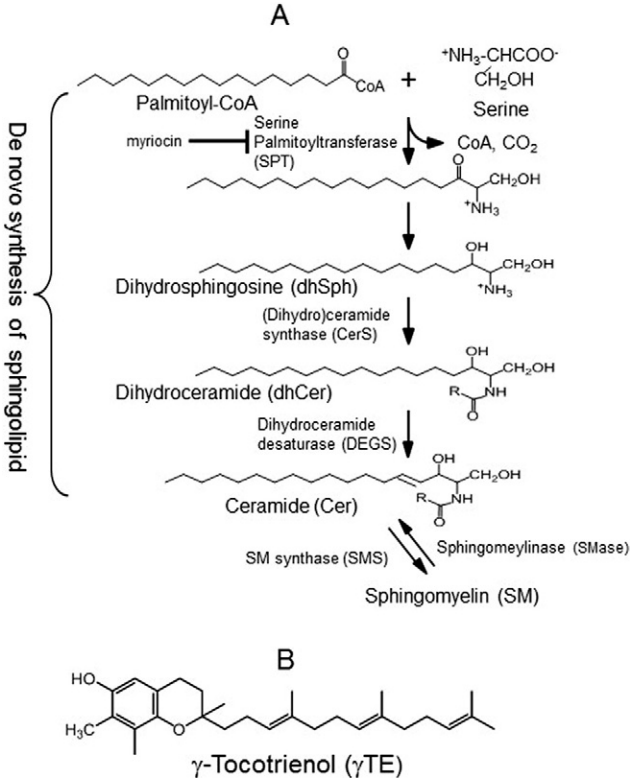

Fig. 1. (A) *De novo* biosynthesis pathway of sphingolipids and interconversion of ceramides and sphingomyelins. R-C(O)-: $C_{16:0}$-, $C_{18:0}$-, $C_{20:0}$-, $C_{22:0}$-, $C_{24:0}$-, $C_{24:1}$-, $C_{26:0}$- $C_{26:1}$-. (B) The structure of γ-tocotrienol (γTE).

[19,20]. DhSph has been reported to be a potent inducer of apoptosis and autophagy [15,21–23].

Given the regulatory role of sphingolipids, modulation of their cellular levels could have important consequences regarding cell fate. Interestingly, several natural compounds that exhibited anticancer activities have been found to modulate sphingolipids including increase of dhCer in various cancer cells [13,15,17,18,24,25]. For instance, we demonstrated that vitamin E forms gamma-tocopherol (γT) and gamma-tocotrienol (γTE) (Fig. 1B) induced dhCer and dhSph accumulation in prostate and breast cancer cells, and the modulation of sphingolipids played an important role in γT and γTE-induced cell death [15,17,24]. While both vitamin E forms have been shown to suppress tumor development in preclinical models, γTE is stronger than γT in these effects [15,26]. Despite these interesting discoveries, previous studies of γT and γTE on sphingolipids were limited to their effect on total Cers or dhCers, and it is not clear how these vitamin E forms affect individual Cers that are believed to have distinct regulatory roles [4,10–12]. Furthermore, the mechanism underlying sphingolipid modulation or potential molecular targets of γTE have not been identified. Here, we investigate the chronological effect of γTE on sphingolipids using liquid chromatography tandem mass spectrometry (LC–MS/MS) in human colon and breast cancer cells. We also used $^{13}C_3$, ^{15}N–labeled L-serine to trace the effect of γTE on *de novo* synthesis of sphingolipids. Based on these results, we have identified enzyme targets of γTE in sphingolipid metabolism including dihydroceramide desaturase.

2. Materials and methods

2.1. Materials and reagents

γTE (97–99%), a gift from BASF (Ludwigshafen, Germany), was dissolved in DMSO at 100 mM and then diluted to 5 mM in fatty acid-free BSA (10 mg/ml). Sphingolipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA). CHAPS was purchased from Thermo Fisher Scientific. C8-cyclopropenylceramide (C8-CPPC) was purchased from Matreya LLC (Pleasant Gap, PA). Myriocin from *Mycelia Sterilia*, desipramine, GW4869, $^{13}C_3$, ^{15}N–labeled L-serine, N-acetyl cysteine (NAC), dimethyl sulfoxide (DMSO), [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide] (MTT) and all other chemicals were from Sigma (St Louis, MO, USA).

2.2. Cell culture and treatment

Human colon HCT-116 (ATCC® CCL-247™), pancreatic PANC-1 (ATCC® CRL-1469™) and breast MCF-7 (ATCC® HTB-22™) cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were routinely cultured in growth media containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. HCT-116 cells were cultured in McCoy's 5A modified medium. PANC-1 and MCF-7 cells were incubated in Dulbecco's modified eagle medium (DMEM) supplemented with 0.1% insulin. At the time of experiments, cells were seeded in each corresponding medium with 10% FBS at 4×10^4 cells/well in 24-well plates or $7–8\times10^5$ cells per 10-cm dish. Unless otherwise indicated, cells were seeded in 10-cm dishes except MTT assay. After overnight attachment, media were replaced with fresh DMEM containing 1% FBS and γTE or other compounds. All the treatment solutions were freshly prepared for each experiment.

2.3. MTT assay

Cell viability was examined by the estimation of mitochondrial dehydrogenase activity. In living cells, the enzyme reduces MTT to form formazan which was dissolved in DMSO and measured the absorbance at 570 nm by using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA).

2.4. Lipid extraction

Total lipids were extracted as previously described [27]. Briefly, cell pellets were resuspended in methanol/chloroform/water [10:5:1 (v/v/v)] after the addition of internal standard mixture containing 0.5 nmol $C_{12:0}$/$C_{25:0}$-Cers, $C_{12:0}$-SM and C17-Sph/dhSph (Avanti Polar Lipids, Alabaster, AL, USA). The suspension was dispersed fully by tip sonication for 20 s and then incubated overnight at 48 °C. One hundred microliter of solvent was used to determine the amount of total choline-containing phospholipids by an enzymatic colorimetric assay (Wako chemicals, Osaka, Japan) [24]. Seventy-five microliter of 1-M KOH in methanol was added to the rest of the solvent and sonicated for 30 min. After sonication, samples were incubated at 37 °C for 2 h and dried in a nitrogen evaporator.

2.5. Measurement of sphingolipids using liquid chromatography tandem mass spectrometry (LC–MS/MS)

Sphingolipids were measured by an LC–MS/MS method modified based on previous publications [27,28]. Analyses were performed using the Agilent 6460 triple quadrupole mass spectrometer coupled with the Agilent 1200 Rapid Resolution HPLC (Agilent Technologies, Santa Clara, CA, USA) with detection of sphingolipids in positive mode by multiple reaction monitoring (MRM) technique. The HPLC mobile phases consisted of methanol-H_2O-formic acid (74:25:1, v/v/v; RA) and methanol-formic acid (99:1, v/v; RB); both RA and RB contain 5-mM ammonium formate. For measurement of Cers and sphingoid bases, Agilent column XDB-C18 (4.6×50 mm) with particle size of 1.8 μm, was used with isocratic run (100% B) or gradient (0–1 min, 20% B, 10–13 min, 100% B and 15–20 min at 20% B), respectively. For measurement of SMs, Agilent Zorbax XDB-C8 (2.1×50 mm) with particle size of 3.4 μm, was used with gradient (0–1 min, 20% B, 10–20 min, 100% B, 22–30 min, 20% B). The MS/MS parameters were as follows: gas temperature, 325–350 °C; gas flow rate, 7–10 L/min; nebulizer pressure, 45–50 psi; capillary voltage, 3500 V. The fragmentor voltage was 100 V, and collision energy was 12–20 V. Precursor-to-product ion transitions for each sphingolipid were used according to the method of Merrill *et al.* [27].

2.6. Evaluation of de novo synthesis of sphingolipids

HCT-116 cells were treated with either 400-μM $^{13}C_3$, ^{15}N–labeled L-serine alone or with a combination of 400-μM $^{13}C_3$, ^{15}N–labeled L-serine and 20 μM of γTE for 30 min, 2 h, 3 h and 6 h. Lipids were extracted, and *de novo* synthesized sphingolipids were measured using LC–MS/MS.

2.7. In situ dihydroceramide desaturase assay

$C_{8:0}$-dhCer was used as the substrate for DEGS. HCT-116 cells were pretreated with either γTE or C8-CPPC as a positive control, followed by incubation with $C_{8:0}$-dhCer (10 μM) for 1 h. The cells were collected, and lipids were extracted. The levels of products ($C_{8:0}$-Cer and $C_{8:0}$-SM) were detected by LC–MS/MS.

2.8. In vitro dihydroceramide desatuase assay

Microsomes were prepared and used as the DEGS enzyme source for this assay [25]. Livers from male Wistar rats were rinsed in ice-cold PBS and homogenized in buffer (0.25-M sucrose, 10-mM HEPES, 1-mM EDTA, pH 7.4) on ice. The homogenate was first