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Palmitoyl-ceramide accumulation with necrotic cell death in A549 cells, followed by a steep increase in sphinganine content

Mototeru Yamane*

Department of Biochemistry, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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Abstract

Ceramides (Cers) have recently been identified as key signaling molecules that mediate biological functions such as cell growth, differentiation, senescence, apoptosis, and autophagy. However, the functions of Cer accumulation in necrotic cell death remain unknown. The aim of this study was to clarify the relationship between Cer accumulation with inhibition of the conversion pathway of Cer and concomitant necrotic cell death. In order to minimize the effect of apoptosis against necrotic cell death, A549 cells having the inhibiting effect of caspase 9 by survivin were used in this study. Consequently, Cer accumulation in A549 cells would likely be associated with a pathway other than the mitochondrial caspase-dependent pathway of apoptosis. Here, we showed that the dual addition of a glucosyl-Cer synthase inhibitor and a ceramidase inhibitor to A549 cell culture induced palmitoyl-Cer accumulation with Cer synthase 5 expression and necrotic cell death with lysosomal rupture together with leakage of cathepsin B/alkalization after 2–3 h, although it is unknown in this study whether the necrotic cell death was caused by the lysosomal rupture. This Cer accumulation was followed by a steep increase in sphinganine base levels via the activation of serine palmitoyltransferase activity brought about by the increase in palmitoyl-coenzyme A concentration as a substrate after 5–6 h. The increase in palmitoyl-coenzyme A concentration was achieved by activation of the fatty acid synthetic pathway from acetyl coenzyme A.

Keywords: Sphinganine; Palmitoyl-ceramide; Necrosis; DL-PDMP; D-NMAPPD; A549 cells.

1. Introduction

Ceramides (Cers) have recently been identified as key signaling molecules that mediate biological functions such as cell growth, differentiation, senescence, apoptosis, and autophagy. Cers, the central molecule involved in sphingolipid biosynthesis, can be generated through the action of ceramide

Abbreviations used: Cer, ceramide; CerS, ceramide synthase; SPT, serine palmitoyltransferase; C16:0-Cer, palmitoyl-ceramide; d18:0, sphinganine; d18:1, sphingosine; Ser, Serine; GlcCer, glucosylceramide; SM, sphingomyelin; DL-PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; D-NMAPPD, N-[(1R,2R)-2-hydroxy-1-(hydroxy-methyl)-2-(4-nitrophenyl)ethyl]tetradecanamide; C16:0-CoA, palmitoyl-coenzyme A; C2:0-CoA, acetyl-coenzyme A; [D_7]d18:0, D-erythro-sphinganine-D7; [D_7]d18:1, D-erythro-sphingosine-D7; d18:1-[D_{31}]C16:0-Cer, N-palmitoyl [D_{31}]-D-erythro-sphingosine; IS, internal standard; L-[2,3,3-D_3]Ser, L-serine-2,3,3-D_3 ; [1,2,3,4-$^{13}C_4$]C16:0 acid, palmitic acid-1,2,3,4-$^{13}C_4$; [2-^{13}C]C2:0 acid, sodium acetate-2-^{13}C; ([$^{13}C_{16}$]C16:0-CoA, palmitoyl-$^{13}C_{16}$ coenzyme A; acridine orange, 3,6-Bis(dimethylamino) acridine hydrochloride; Myriocin, 2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxo-6-eicosenoic acid; SPTLC, SPT-long chain base subunit; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; MAM, mitochondria-associated membrane; SIM, selected-ion monitoring; LDH, lactate dehydrogenase; DMSO, dimethylsulfoxide; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LMP, lysosomal membrane permeabilization; FATP1, fatty acid transport protein 1; 4-HPR, N-(4-hydroxyphenyl)retinamide; CHOP, CAAT/enhancer binding protein homologous protein; LC3, microtubule-associated protein 1 light chain 3B; CathB, cathepsin B; Lys, lysosomes; ER, endoplasmic reticulum.

* Fax: +81 3 3739 9945.
E-mail address: m-yamane@tokyo-med.ac.jp, CBC01664@nifty.com.

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synthases (CerS) in the *de novo* synthesis pathway via serine palmitoyltransferase (SPT) or the salvage pathway. Six different CerS (CerS1 – 6) have been described, each utilizing fatty acyl CoAs of relatively defined chain lengths for N-acylation of sphingoid long chain base [sphinganine (d18:0) and sphingosine (d18:1)]. CerS1 synthesizes mostly C18:0-/C18:1-Cer, CerS2 synthesizes preferentially C22:0-/C24:0-/C24:1-Cer, CerS3 synthesizes very long chain Cers (>C26:0-Cer), CerS4 synthesizes mostly C18:0-/C20:0-/C24:0-Cer, and CerS5/6 synthesizes mainly C14:0-/C16:0-Cer [1].

In recent years, the formation of Cer channel via the interaction with Bax in the mitochondrial outer membrane, followed by the release of cytochrome c into the cytoplasm for the activation of the mitochondrial pathway of apoptosis and a direct Cer-autophagosomal membrane interaction for mitophagy have been reported [2,3]. However, the functions of Cer accumulation in necrotic cell death remain unknown. The aim of this study was to clarify the relationship between Cer accumulation with inhibition of the conversion pathway of Cer and concomitant necrotic cell death. In order to minimize the influence of apoptosis against necrotic cell death, A549 cells having the inhibiting effect of caspase 9 brought about by survivin were used in this study. Consequently, active caspase 3 expression with palmitoyl-Cer (C16:0-Cer) accumulation in A549 cells was not detected by the inhibiting effect of caspase 9 activation by survivin in the cells [4,5], and C16:0-Cer accumulation in A549 cells would likely be associated with a pathway other than the mitochondrial caspase-dependent pathway including the Bax/Bak activation of apoptosis. Previously, we showed that a high concentration of DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol [DL-PDMP, an inhibitor of glucosyl(Glc)-Cer synthase] [6] in A549 cell culture caused massive autophagy with endoplasmic reticulum stress and C16:0-Cer accumulation via CerS5 protein expression in A549 cells, followed by autophagic cell death 24 h after treatment [5]. Here, we showed that the dual addition of DL-PDMP and N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]tetradecanamide (D-NMAPPD, an inhibitor of ceramidase) [7] to A549 cell culture induced an additional C16:0-Cer accumulation with CerS5 expression and necrotic cell death with lysosomal rupture together with leakage of cathepsin B/alkalization after 2–3 h. This Cer accumulation was followed by a steep increase in d18:0 base levels via the activation of SPT activity brought about by the increase in palmitoyl-coenzyme A (C16:0-CoA) concentration as a substrate after 5–6 h.

2. Materials and methods

2.1. Materials

D-erythro-sphinganine-D_7 ([D_7]d18:0), D-erythro-sphingosine-D_7 ([D_7]d18:1), and N-palmitoyl [D_{31}]-D-erythro-sphingosine (d18:1-[D_{31}]C16:0-Cer) as internal standards (ISs) labeled with stable isotopes or 1-deoxysphinganine were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). L-serine-2,3,3-D_3 (L-[2,3,3-D_3]Ser) as the tracer labeled with stable isotopes was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Palmitic acid-1,2,3,4-$^{13}C_4$ ([1,2,3,4-$^{13}C_4$]C16:0 acid) or sodium acetate-2-^{13}C ([2-^{13}C]C2:0 acid) as the tracer labeled with stable isotopes, palmitoyl-$^{13}C_{16}$ coenzyme A ([$^{13}C_{16}$]C16:0-CoA) lithium salt as the IS, palmitoyl-coenzyme A (C16:0-CoA) lithium salt, sucrose monolaurate, pyridoxal 5′-phosphate hydrate, fumonisin B(1) and bovine albumin (essentially fatty acid free) were obtained from Sigma–Aldrich, Co. (St. Louis, MO, USA). NEFA C (kit for the measurement of free fatty acid content), 3,6-Bis(dimethylamino) acridine hydrochloride solution (acridine orange solution, 1 mg/ml water), Celite, 10% ammonia aqueous solution, sodium tetrahydroborate (sodium borohydride), dithiothreitol, and lithium dodecyl sulfate were purchased from Wako (Osaka, Japan). D-NMAPPD as an inhibitor of ceramidase and 2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxo-6-eicosenoic acid (myriocin) as an inhibitor of SPT were purchased from Cayman Chemical (Ann Arbor, MI, USA). DL-PDMP was obtained from Biomol Research Labs. (Plymouth Meeting, PA, USA). Anti-Cer synthase 5 (anti-LASS5) antibody (PAB8802) was procured from Abnova (Taipei, Taiwan). Anti-Cer synthase 6 (anti-LASS6) antibody (GTX51627) was procured from Genetex, Inc. (Irvine, CA, USA). Anti-SPT-long chain base subunit-1 (anti-SPTLC1) antibody and anti-SPT-long chain base subunit-2 (anti-SPTLC2) antibody were obtained from Acris Antibodies GmbH (Herford, Germany). Anti-SPT-long chain base subunit-3 (anti-SPTLC3) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. A549 cell culture, induction of Cer accumulation and tracer experiments

A549 cells (human lung adenocarcinoma cell line) were grown in humidified air with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (including 8.5 μM free fatty acids) prepared from Sigma D5796 including 400 μM L-Ser, containing fetal bovine serum (FBS) at the concentration of 10% (v/v), at 37 °C. The induction of Cer accumulation or tracer experiments were usually initiated 1 day after subculture below 90% confluence. Overgrown cells were unsuitable for obtaining the desired effects. In the tracer experiments, 1180 μM L-[2,3,3-D_3]Ser, 5.6 mM [2-^{13}C]C2:0 acid or bovine serum albumin (BSA) binding 2.34–130 μM [1,2,3,4-$^{13}C_4$] C16:0 acid in the culture medium was used. Binding of [1,2,3,4-$^{13}C_4$] C16:0 acid to BSA was performed following the method of Spector and Hoak [8]. Briefly, 100 μM [1,2,3,4-$^{13}C_4$] C16:0 acid in 10 ml of hexane was mixed with 1 g of Celite, and the mixture was evaporated dry under reduced pressure. Celite coated with fatty acid was mixed with 0.68 g of fatty acid-free BSA in 40 ml of Sigma D5796 medium, and the mixture was stirred for 30 min at room temperature. The mixture was centrifuged at 600× *g* for 5 min. The supernatant was filtered through a filter paper and Millex GS (Millipore, Billerica, MA, USA). The concentration of BSA binding C16:0 acid in the supernatant was measured using a NEFA C kit.