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Visualization of ceramide channels in lysosomes following endogenous palmitoyl-ceramide accumulation as an initial step in the induction of necrosis

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ABSTRACT

In this study, we showed that the dual addition of glucosyl ceramide synthase and ceramidase inhibitors to A549 cell culture led to the possibility of ceramide channel formation via endogenous palmitoyl-ceramide accumulation with an increase in cholesterol contents in the lysosome membrane as an initial step prior to initiation of necrotic cell death. In addition, the dual addition led to black circular structures of 10–20 nm, interpreted as stain-filled cylindrical channels on transmission electron microscopy. The formation of palmitoyl-ceramide channels in the lysosome membrane causes the liberation of cathepsin B from lysosomes for necrotic cell death. On the other hand, necrotic cell death in the dual addition was not caused by oxidative stress or cathepsin B activity, and the cell death was free from the contribution of the translation of Bax protein to the lysosome membrane.

1. Introduction

In recent years, the formation of ceramide (Cer) channels that initiated apoptosis on their own or via interaction with BCL2-associated X protein (Bax) in the outer mitochondrial membrane, followed by the release of cytochrome c was firmly established [1–3]. On the other hand, direct interaction of mitochondrial Cer with the autophagosomal membrane bound-microtubule-associated protein 1 light chain 3B (LC3B)-II for mitophagy has been reported [4]. However, the functions of endogenous Cer accumulation in necrotic cell death remain unknown. The aim of this study was to clarify the relationship between endogenous palmitoyl-Cer (C16:0-Cer) accumulation with inhibition of the conversion pathway of Cer and concomitant necrotic cell death.

In A549 cells, active caspase 3 expression with C16:0-Cer accumulation in mitochondria was not detected by the blocking effect in the caspase 9 to caspase 3 process by survivin having an inhibitory effect on the activation of caspase 9 [5,6]. Therefore, endogenous C16:0-Cer accumulation in A549 cells would likely be related to a pathway (e.g., the pathway of necrotic cell death) distinct from the mitochondrial caspase-dependent pathway. 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] in A549 cell culture caused massive autophagy with endoplasmic reticulum stress and endogenous C16:0-Cer accumulation via Cer synthase (CerS) 5 protein expression in A549 cells, followed by autophagic cell death 24 h after treatment [6]. Furthermore, we showed that the dual addition of DL-PDMP and N-[(1R,2R)-2-hydroxy-1-(hydroxy-methyl)-2-(4-nitrophenyl)ethyl]tetradecanamide (D-NMAPPD, an inhibitor of ceramidase) to A549 cell culture induced additional endogenous C16:0-Cer accumulation with CerS5 expression and necrotic cell death with lysosomal rupture along with the leakage of cathepsin B/alkalization 2–3 h afterwards [7]. If C16:0-Cer channels were formed in the lysosome membrane with endogenous C16:0-Cer accumulation via the activation of CerS5 and the inhibition of lysosomal acid ceramidase by D-

Abbreviations: Cer, ceramide; Bax, BCL2-associated X protein; LC3B, autophagosomal membrane bound-microtubule-associated protein 1 light chain 3B; C16:0-Cer, palmitoyl-Cer; DL-PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; CerS, ceramide synthase; GlcCer, glucosyl ceramide; D-NMAPPD, N-[(1R,2R)-2-hydroxy-1-(hydroxy-methyl)-2-(4-nitrophenyl)ethyl]tetradecanamide; [D_7]d18:0, D-erythro-sphinganine-D_7; [D_7]d18:1, D-erythro-sphingosine-D_7; d18:1-[D_{31}]C16:0-Cer, N-palmitoyl [D_{31}]-D-erythro-sphingosine; ISs, internal standards; CA-74Me, [(2S,3S)-3-Propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester; NAC, N-acetyl-L-cysteine; acridine orange, 3,6-Bis(dimethylamino) acridine hydrochloride; LAMP-2, lysosome-associated membrane protein 2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence reagent; APCI, atmospheric pressure chemical ionization; MS, mass spectrometry; d18:0, sphinganine; d18:1, sphingosine; SIM, selected ion monitoring; DMSO, dimethyl sulfoxide; DAPI, 4′,6-diamidino-2-phenylindole; LMP, lysosomal membrane permeability

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NMAPPD, this possibility would be of interest as a function of the liberation of cathepsin B from lysosomes causing necrotic cell death via C16:0-Cer channels in the lysosome membrane distinct from the mitochondrial caspase-dependent pathway of apoptosis.

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 were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). [(2S,3S)-3-propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074Me as an inhibitor of cathepsin B), N-acetyl-L-cysteine (NAC as an inhibitor of oxidative stress), 3,6-Bis(dimethylamino) acridine hydrochloride (acridine orange) solution (1 mg/mL water), 10% ammonia aqueous solution, cholesterol, dithiothreitol, and lithium dodecyl sulfate were purchased from Wako (Osaka, Japan). D-NMAPPD as an inhibitor of ceramidase was purchased from Cayman Chemical (Ann Arbor, MI). DL-PDMP was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). β-cholestanol was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2. A549 cell culture, induction of Cer accumulation

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-serine, containing fetal bovine serum (FBS) at a concentration of 10% (v/v), at 37 °C. The induction of Cer accumulation was usually initiated 1 day after subculture below 90% confluence. Overgrown cells were unsuitable for obtaining the desired effects.

For the induction of Cer accumulation, 200 μM DL-PDMP and 65 μM D-NMAPPD in the culture medium were incubated for 2–5 h. For the inhibition of cathepsin B or oxidative stress, 20 μM CA-074Me or 5000 μM NAC was added to the culture medium.

2.3. Isolation of the lysosome-rich fraction from A549 cells

A549 cells were collected after incubation for 2 h by scraping with the culture medium and rinsing two times with Hank's balanced salt solution. Isolation of the lysosome-rich fraction from A549 cells was performed with Lysosome Enrichment Kit for Tissue and Cultured Cells (Thermo Scientific, Rockford, IL). After A549 cells had been added to 250 μM of Lysosome Enrichment Reagent A, the mixture was mixed by Vortex for 5 s, incubated on ice for 2 min, and treated by mild sonication (6–9 W of power) for 10 s. The suspension was mixed with 250 μM of Lysosome Enrichment Reagent B and centrifuged at 500 × *g* for 10 min at 4 °C. The supernatant was overlayed on the top of a prepared discontinuous density gradient and gradient centrifuged at 145,000 × *g* for 2 h at 4 °C. The lysosome-rich band was mixed with five volumes of phosphate buffered saline (PBS) and rinsed by centrifugation at 18,000 × *g* for 30 min. The residue was rinsed with PBS in a similar manner.

2.4. Western blotting with LAMP-2/Bax/β-actin antibodies

A549 cells were incubated for 2 h, scraped with the culture medium and rinsed two times with Hank's balanced salt solution. The cells or the suspension of the lysosome-rich fraction in 1.0 mL of 0.85% sodium chloride was mixed with 100 μL of 100% (1.0g/mL aqueous solution) trichloroacetic acid solution and left for 30 min at 0 °C. The mixture was centrifuged at 1500 × *g* for 5 min. The pellet was mixed with 80 μL of 9 M urea/2% Triton X100/1% dithiothreitol (DTT) and the mixture was treated with ultrasonic waves for 30 s. The mixture was mixed with 20 μL of 10% lithium dodecyl sulfate and made basic with 1 M Tris under ultrasonic waves for 30 s.

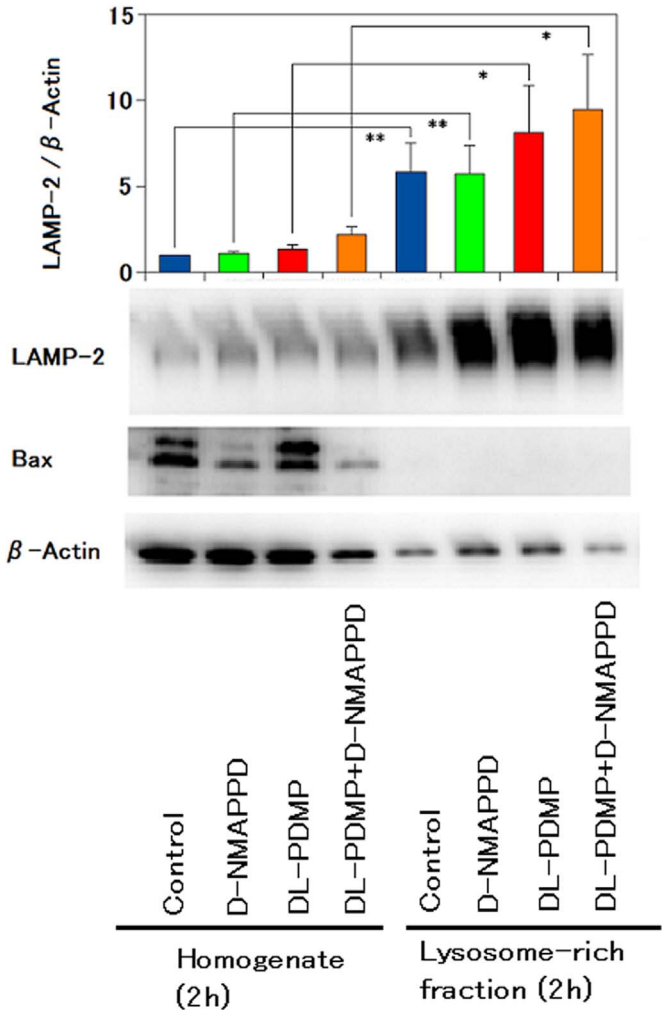

Fig. 1. The analysis of LAMP-2, Bax, and β-actin in the homogenate or lysosome-rich fraction from A549 cells by Western blotting. Western blotting analysis was achieved as described in Methods. LAMP-2/β-actin was high in the lysosome-rich fraction and LAMP-2 protein was concentrated in the lysosome-rich fraction. However, Bax protein was not detected in the lysosome-rich fraction. Data are presented as the mean values ± S.D. of three independent experiments;*P < 0.05 and **P < 0.01 as compared with the control or the individual addition.

Equal amounts of proteins were loaded onto the gels, separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). The membranes were probed with primary antibodies such as anti-LAMP-2 (clone H4B4; 1/1000 dilution) antibody, anti-BAX (clone N-20; 1/1000 dilution) antibody, and anti-β-actin (clone C4; 1/1000 dilution) antibody (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 16 h at 4 °C. Immunoreactive proteins were detected with horseradish peroxidase-conjugated second antibody (Jackson, West Grove, PA; 1:25000 dilution) for 1 h at room temperature and an enhanced chemiluminescence reagent (ECL) (Millipore). Densitometry was performed using a Molecular Imager, ChemiDoc XRS System (Bio-Rad, Richmond, CA).

2.5. Lipid extraction of from A549 cells or the lysosome-rich fraction after various additions

A549 cells were collected each time by scraping with the culture medium and rinsing two times with Hank's balanced salt solution. Cells