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Sphingomyelin synthase 1 suppresses ceramide production and apoptosis post-photodamage

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Abstract

The role of sphingomyelin synthase 1 (SMS1), the Golgi membrane isoform of the enzyme, in ceramide metabolism and apoptosis after photodamage with the photosensitizer Pc 4 (PDT) is unclear. In the present study, using electrospray ionization/double mass spectrometry, we show that in Jurkat cells overexpressing SMS1, increases in ceramides were lower than in empty-vector transfectants post-PDT. Similarly, the responses of dihydroceramides and dihydrosphingosine, precursors of ceramide in the *de novo* synthetic pathway, were attenuated in SMS1-overexpressor after photodamage, suggesting the involvement of the *de novo* pathway. Overexpression of SMS1 was associated with differential regulation of sphingomyelin levels, as well as with the reduced inhibition of the enzyme post-treatment. Concomitant with the suppressed ceramide response, PDT-induced DEVDase activation was substantially reduced in SMS1-overexpressors. The data show that overexpression of SMS1 is associated with suppressed ceramide response and apoptotic resistance after photodamage.

Keywords: Apoptosis; Ceramide; Electrospray ionization/double mass spectrometry; PDT; Phthalocyanine Pc 4; Sphingomyelin synthase

Ceramide is a key metabolite in both anabolic and catabolic pathways of sphingolipids [1,2]. *De novo* sphingolipid biosynthesis begins with serine palmitoyltransferase (SPT)-dependent condensation of palmitoyl CoA and L-serine, resulting in the synthesis of 3-ketodihydrosphingosine. In subsequent reactions dihydrosphingosine, dihydroceramide, and *de novo* ceramide are formed, and the latter is acted upon by sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS), to give rise to sphingomyelin and glucosylceramide, respectively.

Using pharmacologic and genetic approaches, we have demonstrated that *de novo* sphingolipids are involved in initiation of apoptosis after photodamage with Pc 4 (PDT) [3,4]. We have shown that in the absence of SPT upregulation *de novo* ceramide accumulates, while SMS and GCS are inhibited post-PDT [5]. These findings support the idea that PDT induces *de novo* ceramide accumulation by inhibition of SMS and GCS. A correlation between apoptotic resistance and increased SMS activity has been shown [6]. In the present study, SMS1, the Golgi

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isoform of the enzyme, [7] was overexpressed in Jurkat cells to test directly the involvement of SMS1 in ceramide production and apoptosis post-PDT.

Methods

Materials. The phthalocyanine photosensitizer Pc 4, HOSiPcOS $i(CH_3)_2(CH_2)_3N(CH_3)_2$, was from Dr. Malcolm E. Kenney (Case Western Reserve University).

Cell culture and treatment. Jurkat, clone E6-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin, and were maintained at 37 °C in a 5% CO_2 atmosphere. For PDT experiments, cells were treated in growth medium and all incubations were performed at 37 °C in a 5% CO_2 atmosphere. After overnight incubation with Pc 4 (200 nM), cells were irradiated with red light (2 mW/cm^2; $\lambda_{max} \sim 670$ nm) using a light-emitting diode array light source (EFOS) at various fluences at room temperature. The fluences of 135, 270, and 400 mJ/cm^2 were used for the low, mid and high PDT dose, respectively. Two hours post-PDT, cells were harvested, washed with PBS, and further processed for various analyses. For mass spectrometric analysis, cells were washed twice with PBS, resuspended in 100 μl ethyl acetate/methanol (1:1, v/v), dried under nitrogen, and shipped overnight on dry ice to the Lipidomics Core (Charleston, SC) for further processing.

Stable transfection of Jurkat cells. The SMS1 gene construct SMS1-V5/pcDNA3.1 was kindly provided by Dr. Holthuis (Utrecht University, Netherlands) and was described previously [7]. Jurkat cells (10^7) were transfected with empty-vector pcDNA3.1 (Invitrogen) and SMS1 plasmid by electroporation (400 V, 960 μF capacitor, single pulse) using the Micropulser Electroporator (Bio-Rad). Fifty micrograms of each non-linearized DNA was transfected. Two independent transfections were performed for each plasmid. Similarly, transfections of Jurkat cells (5×10^5) using Lipofectamine 2000 (2.5 μl; Invitrogen) were carried out for each plasmid (2 μg) according to manufacturer's instructions. Forty-eight hours after transfections, the cells were grown in the regular medium supplemented with 800 μg/ml Geneticin (Invitrogen), which was replenished every 4 days up to 1 day prior to an experiment. Pooled transfectants were expanded for three-four weeks, and tested for SMS activity.

Electrospray ionization/double mass spectrometry analysis. Following extraction, sphingolipids were separated by high performance liquid chromatography, introduced to electrospray ionization source and then analyzed by double mass spectrometry (Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer), which allows simultaneous determination of various sphingolipids, including ceramide, dihydroceramide, and sphingomyelin species, as well as dihydrosphingosine and sphingosine-1-phosphate [8]. Specifically, cells (5×10^6) were fortified with the internal standards (C17-base-D-erythro-sphingosine, C17-sphingosine-1-phosphate, *N*-palmitoyl-D-erythro-C13-sphingosine, and C17-D-erythro-sphingosine), and extracted with ethyl acetate/iso-propanol/water (60/30/10, v/v). After evaporation and reconstitution in 100 μl of methanol, samples were injected into the HP1100/TSQ 7000 LC/MS system and gradient-eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3 μm particle size column, with 1 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase. Peaks corresponding to the target analytes and internal standards were collected and processed using the Xcalibur software system. Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the internal standards. For the calibration curves, the target analyte/internal standard peak area ratios were plotted against analyte concentration. The target analyte/internal standard peak area ratios from the samples were similarly normalized to their respective internal standards and compared to the calibration curves, using a linear regression model. The amounts of sphingosine-1-phosphate were below detection limits (1 pmole per total extracted sample), and are not reported. The method is referred to in the remaining text as mass spectrometry.

Preparation of microsomal membranes. SPT, SMS, and GCS activities. Enzyme activities were measured in microsomal membranes prepared from cells treated with PDT as described previously [5,9]. The SPT activity assay [10] is based on the ability of SPT to incorporate L-[^{3}H]serine (GE Healthcare) into its product 3-ketodihydrosphingosine. The [^{3}H]3-ketodihydrosphingosine was extracted and the radioactivity was measured by scintillation counting. The assay of SMS and GCS activities [11] is based on the ability of SMS and GCS to incorporate the fluorogenic derivative of C6-ceramide, (*N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine (C6-NBD-ceramide; Avanti), into sphingomyelin and glucosylceramide, respectively. C6-NBD-ceramide-labeled lipid products were extracted, separated by thin layer chromatography (TLC) using chloroform/methanol/water (65/25/4, v/v), and their fluorescence was detected and quantified by the STORM 860 imaging system (GE Healthcare).

DEVDase activity. As described previously [5], DEVDase activity was determined in cytosol by an assay based on the enzyme's ability to cleave the fluorogenic derivative 7-amino-4-methylcoumarin (AMC; Biomol) of the tetrapeptide substrate *N*-acetyl-Asp-Glu-Val-Asp (DEVD). The released fluorescence of the cleaved DEVD substrate was measured in a F-2500 Hitachi spectrofluorometer (380 nm excitation and 460 nm emission).

Results

Using diacylglycerol kinase assay and [^{14}C]serine metabolic labeling, we have shown that PDT triggers increase in ceramide mass and in *de novo* ceramide accumulation, respectively [5,12–14]. Here we used mass spectrometry for the first time to compare the effects of photodamage in SMS1-overexpressors and empty-vector transfectants on the mass of sphingolipids. For all sphingolipids the following trends were observed: (i) in both cell types resting levels were higher for C16-, C24-, and C24:1-sphingolipids, than for other sphingolipids; (ii) in SMS1-overexpressors, basal levels of sphingolipids were higher than in their control counterparts (e.g., basal levels of C16-, C24-, and C24:1-ceramide were 67–93% higher in SMS1-overexpressors than in empty-vector transfectants); (iii) the responses to photodamage in empty-vector transfectants were prevented in SMS1-overexpressors. Specifically, in empty-vector transfectants, compared to Pc 4-controls, the mass of C16-ceramide was increased by 2.5-, 2.6-, and 2.9-fold after low, mid and high PDT dose, respectively (Fig. 1A). The production of C24- and C24:1-ceramide was also markedly increased in empty-vector transfectants after corresponding PDT doses (Fig. 1B and C). Similar trends were detected in empty-vector transfectants post-PDT for other ceramides (C14-, C18-, C18:1-, C20-, C20:1-, C22-, C22:1-, C26-, and C26:1-ceramide; not shown). In contrast, in SMS1-overexpressors, the mass of C16-ceramide was at resting levels after the low PDT dose, whereas at mid and high PDT dose the lipid levels were increased only by 1.4- and 1.5-fold, respectively (Fig. 1A). Similar trends were observed in SMS1-overexpressors after PDT for C24- and C24:1-ceramide (Fig. 1B and C), and other ceramides (not shown). Thus, exposure of SMS1-overexpressors to PDT led to either no ceramide response or to a suppressed ceramide production compared to their control counterparts.

Dihydroceramides and dihydrosphingosine, precursors of ceramide in the *de novo* pathway, were also detected by mass spectrometry. Treatment of either cell type with