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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

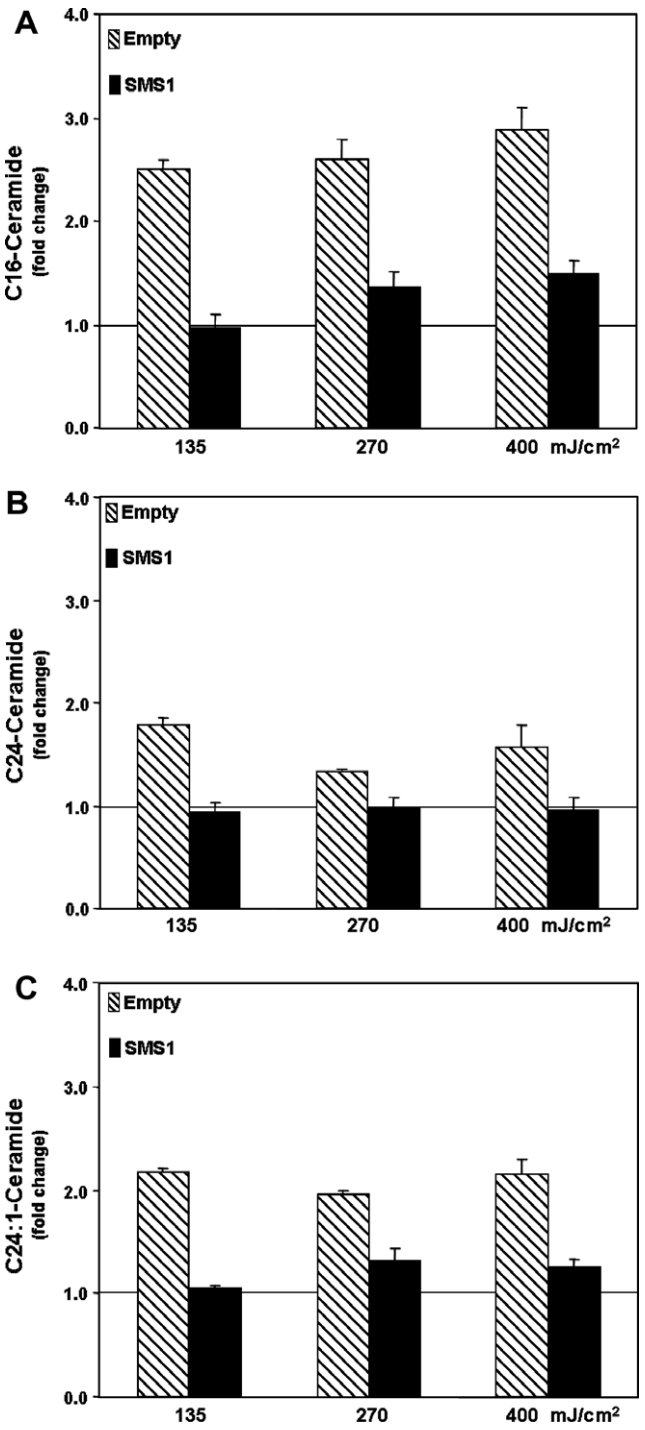

Fig. 1. Mass spectrometric analysis reveals PDT-induced changes in ceramides in empty-vector transfected and SMS1-overexpressing Jurkat cells. (A) C16-ceramide; (B) C24-ceramide; (C) C24:1-ceramide. After overnight preincubation with Pc 4 (200 nM), cells were irradiated at the indicated light fluences (mJ/cm^2), and then incubated for 2 h. After cell harvesting and lipid extraction, ceramide levels were determined by mass spectrometry (see Methods). The data are expressed as -fold changes relative to Pc 4-controls and are shown as means ± SEM of 2–4 values. The resting Pc 4-control values (fmoles/mg; $n = 5$–6) for C16-, C24-, and C24:1-ceramide were: in empty-vector transfectants, 390 ± 130, 140 ± 30, and 130 ± 30, respectively; in SMS1-overexpressors, 650 ± 200, 270 ± 30, and 240 ± 50, respectively. This and subsequent figures: empty, empty-vector transfectants; SMS1, SMS1-overexpressing Jurkat cells.

PDT led to increases in C16-, C24-, and C24:1-dihydroceramide, except that the response in SMS1-overexpressors was reduced (Fig. 2A–C). In particular, in empty-vector transfectants the production of C24:1-dihydroceramide was increased by 3.0-, 3.6-, and 7.1-fold after corresponding PDT doses (Fig. 2C). Moreover, in the same cell type the formation of dihydrosphingosine was increased by 139%, 40%, and 26% after corresponding PDT doses, whereas in SMS1-overexpressors the levels of dihydrosphingosine were near or below resting levels under the same conditions (Fig. 2D). Overall, similar to ceramide, the production of dihydroceramides and dihydrosphingosine was suppressed in SMS1-overexpressors compared to empty-vector transfectants. The data imply that photodamage-induced increases in *de novo* sphingolipids are prevented by overexpression of SMS1.

Sphingomyelin, a product of sphingomyelin synthase, was also analyzed by mass spectrometry. Exposure of empty-vector transfected Jurkat cells to the low PDT dose increased C16-sphingomyelin mass by 69% (Fig. 3A). However, at higher PDT doses, C16-sphingomyelin response returned back to near-resting levels (Fig. 3A). In contrast, in SMS1-overexpressors, C16-sphingomyelin levels were below resting levels at all three PDT doses (Fig. 3A). Similar findings were obtained in both cell types for C24- and C24:1-sphingomyelin (Fig. 3B and C), as well as other sphingomyelins (i.e., C14-, C18-, C18:1-, C20-, C20:1-, C22-, C22:1-sphingomyelin; not shown). Taken together, in contrast to empty-vector transfectants, the mass of sphingomyelins was below basal levels in SMS1-overexpressors after photodamage.

We have shown that PDT induces inhibition of SMS and GCS without affecting SPT activity in Jurkat parental cells [5]. The activities of these enzymes were determined in microsomes isolated from Pc 4- or PDT-treated empty-vector transfectants and SMS1-overexpressing Jurkat cells. Compared to empty-vector transfectants, overexpression of SMS1 led to a 3.8-fold increase in SMS activity of Pc 4-controls. Resting GCS levels were similar in both cell types, whereas basal SPT activity was reduced by 42% in SMS1-overexpressors. In empty-vector transfectants SMS activity was inhibited by 77% and 81% at the mid and high PDT dose, respectively (Fig. 4A). In SMS1-overexpressors the enzyme was inhibited to a lesser extent, i.e., by 55% and 36% at corresponding PDT doses (Fig. 4A). In addition, in empty-vector transfectants GCS activity was inhibited by 58% and 69% at the mid and high PDT dose, respectively (Fig. 4A). In SMS1-overexpressors, at the mid PDT dose, GCS was inhibited by 52%, whereas at the high PDT dose the enzyme was activated by 180% (Fig. 4A). Next, we tested the effects of PDT on SPT activity in the two cell types. In either cell type there was no significant effect of PDT on SPT (Fig. 4A). Hence, compared to empty-vector transfectants, in SMS1-overexpressors SMS activity was less inhibited after photodamage, GCS was activated at the high PDT dose, and the resting SPT activity was reduced, whereas PDT had no effect on SPT in either cell type.

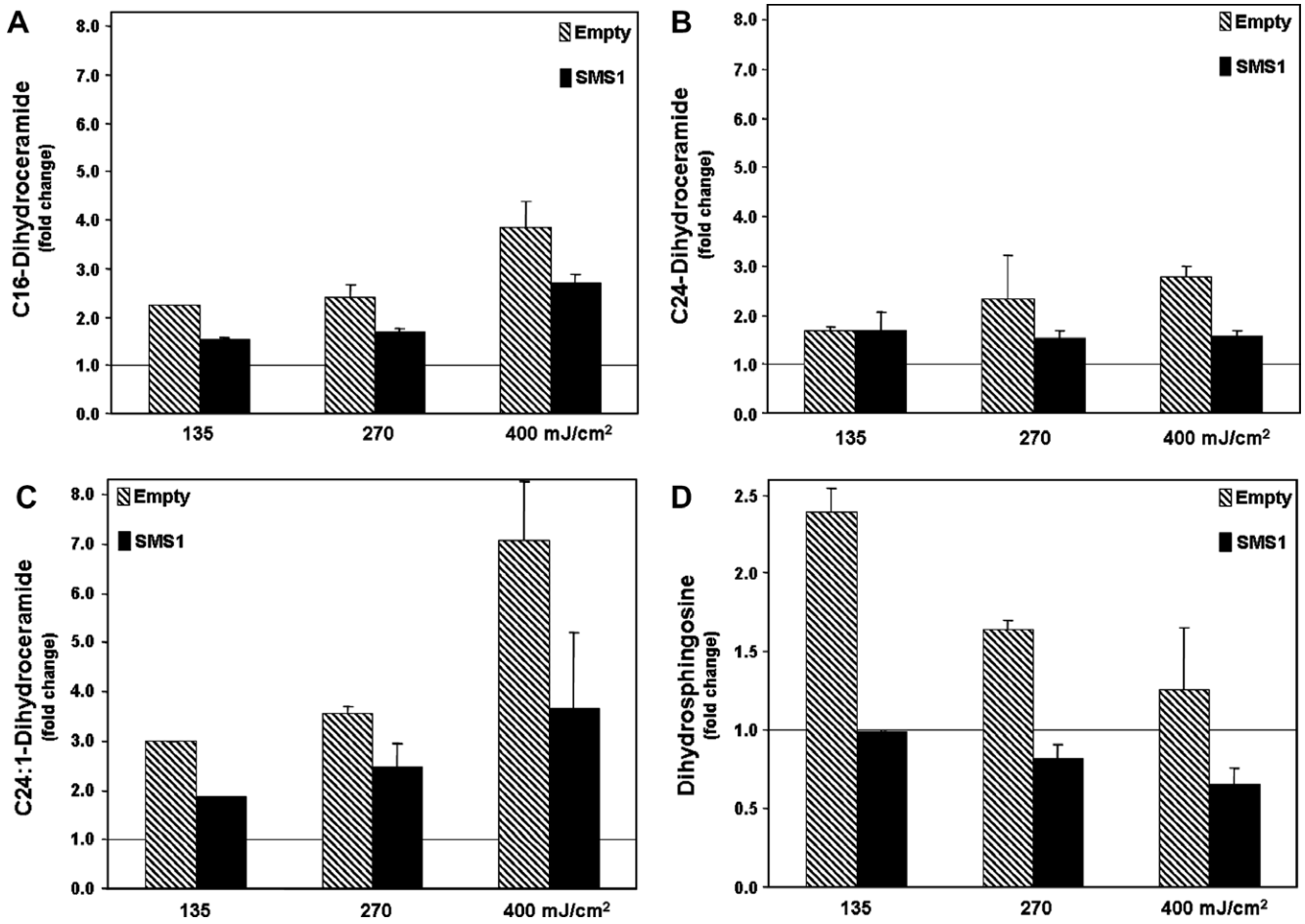

Fig. 2. Mass spectrometric analysis identifies PDT-induced changes in the precursors of *de novo* ceramide in empty-vector transfected and SMS1-overexpressing Jurkat cells. (A) C16-dihydroceramide; (B) C24-dihydroceramide; (C) C24:1-dihydroceramide; (D) dihydrosphingosine. The data are expressed as -fold changes relative to Pc 4-controls and are shown as means ± SEM of 2–4 values (with the exception of the C24:1-dihydroceramide values for the low PDT dose). The resting Pc 4-control values (fmoles/mg; $n = 3$–6) for C16-, C24-, and C24:1-dihydroceramide, and dihydrosphingosine were: in empty-vector transfectants, 30 ± 2, 5 ± 2, 2 ± 0, and 2 ± 0, respectively; in SMS1-overexpressors, 50 ± 5, 7 ± 1, 6 ± 1, and 4 ± 1, respectively.

We have already shown that Jurkat cells undergo apoptosis post-PDT [4,15]. In this study we tested whether PDT-induced DEVDase activation is affected by overexpression of SMS1. Treatment of empty-vector transfectants with PDT (200 nM + 135, 270, and 400 mJ/cm^2) led to 2.4-, 11.7-, and 24.8-fold increases in DEVDase activity, respectively (Fig. 4B). Under the same conditions in SMS1-overexpressors DEVDase activity was increased by 2.0-, 3.9-, and 6.2-fold, respectively (Fig. 4B). The data demonstrate that PDT induced a dose-dependent DEVDase activation in both cell types but, compared to empty-vector transfectants, the responses were substantially suppressed at the mid and high PDT dose in SMS1-overexpressors.

Discussion

The present study shows for the first time that PDT-induced changes in the mass of sphingolipids during apoptosis are prevented in SMS1-overexpressors. C16-ceramide is the most abundant ceramide species, as well as other C16-sphingolipids, in Jurkat cells. Reportedly, C16-ceramide is abundant in tumor cells [16]. C16- and C24-ceramide have been associated with apoptosis [17–19]. Although we show here that in Jurkat cells the levels of a number of ceramides are increased in response to photodamage, only dihydroceramides, in particular C16- and C24:1-dihydroceramide, show dose-response trends supporting some selectivity in favor of these ceramide species. PDT also induced increases in dihydrosphingosine, another precursor from the *de novo* synthesis. The data indicate the involvement of *de novo* pathway in photodamage, a notion which is also supported by our previously published evidence [3–5,15].

Following photodamage, there was a lack of correlation between SMS activity and sphingomyelins in both cell types. In addition to SMS, sphingomyelin levels are regulated by other enzymes, e.g., sphingomyelinase. We have shown that acid sphingomyelinase is inhibited by PDT [20]. The net result of the opposing effects of photodamage-induced inhibition of the two enzymes might be reflected in the observed changes in sphingomyelin levels. In addition, an alternative route of sphingomyelin synthe-

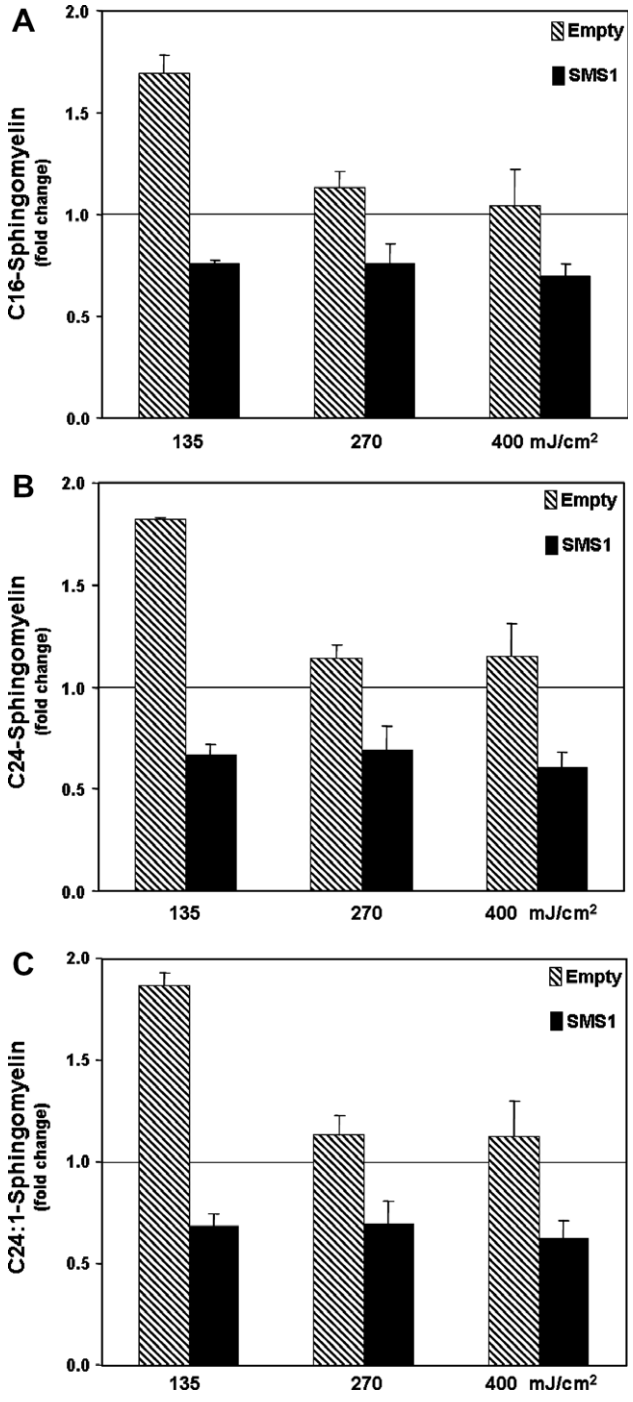

Fig. 3. Mass spectrometric analysis demonstrates PDT-induced changes in sphingomyelins in empty-vector transfected and SMS1-overexpressing Jurkat cells. (A) C16-sphingomyelin; (B) C24-sphingomyelin; (C) C24:1-sphingomyelin. The data are expressed as -fold changes relative to Pc 4-controls and are shown as means ± SEM of 2–4 values. The resting Pc 4-control values (fmoles/mg; $n = 5$–6) for C16-, C24-, and C24:1-sphingomyelin were: in empty-vector transfectants, 9370 ± 1820, 670 ± 170, and 2960 ± 280, respectively; in SMS1-overexpressors, 13,370 ± 2730, 1660 ± 190, and 5480 ± 360, respectively.

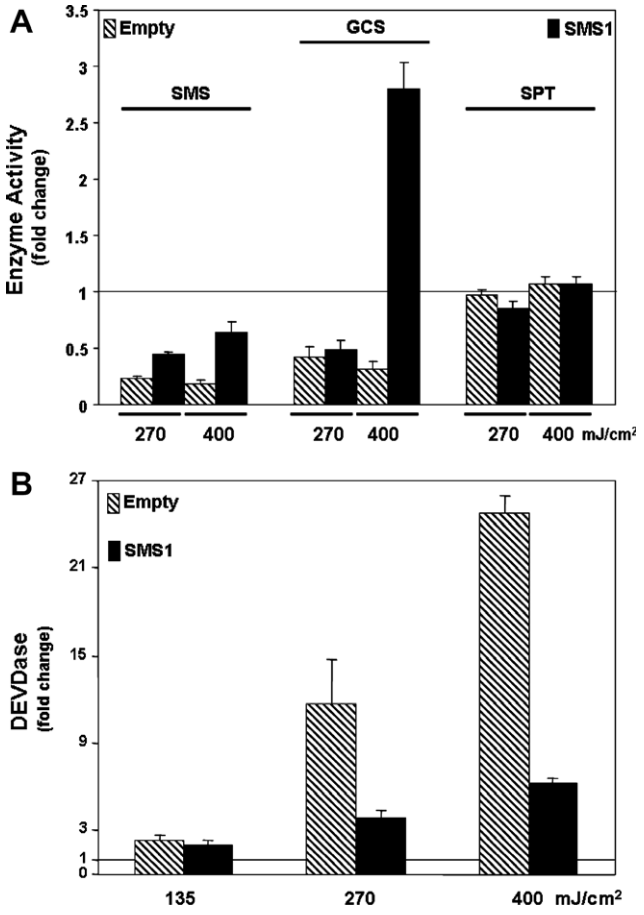

Fig. 4. The effects of PDT on the activities of (A) SMS, GCS, SPT and (B) DEVDase in empty-vector transfected and SMS1-overexpressing Jurkat cells. Jurkat cells were treated overnight with Pc 4 (200 nM), irradiated at the indicated light fluences and then incubated for 2 h. The data are expressed as -fold changes relative to Pc 4-controls and are shown as the means ± SEM of 4–8 values. After the cells were harvested, (A) microsomes were isolated and the activities of the enzymes were assayed; (B) DEVDase activity was measured in cell lysates spectrofluorimetrically using DEVD-AMC as the substrate. The resting Pc 4-control values for SMS and GCS (nmoles/mg/5 min; $n = 4$–5), SPT (nmoles/mg/10 min; $n = 5$), and DEVDase (arbitrary units; $n = 7$) were: in empty-vector transfectants, 76 ± 16, 62 ± 7, 65 ± 23, and 347 ± 49, respectively; in SMS1-overexpressors, 289 ± 89, 63 ± 1, 38 ± 14, and 454 ± 45, respectively.

sis via ethanolamine phosphorylceramide synthase [21] could be a target of photodamage. Overall, the data indicate that the response of sphingomyelin to photodamage is regulated not only by SMS but also other enzymes.

Overexpression of SMS1 leads not only to an increased resting activity of SMS, but also to a changed response of the enzyme to PDT, so that SMS is inhibited to a lesser extent in overexpressors than in their control counterparts. The data suggest that the overexpressed enzyme partly ''escapes'' the photodamage-induced inhibition of the ceramide-to-sphingomyelin conversion. Altered responses of SMS to PDT in SMS1-overexpressors are reflected in suppressed ceramide production after photodamage. Similarly, after PDT, the increases in dihydroceramides are for the most part reduced in SMS1-overexpressors compared to empty-vector transfectants. We have shown the evidence suggesting that PDT-induced inhibition of ceramide-metabolizing enzymes, e.g., SMS, leads to passive accumu-

lation of *de novo* ceramide [5]. Our present data further support the hypothesis since reduced inhibition of SMS after photodamage in SMS1-overexpressors is accompanied by suppressed accumulation of dihydroceramides, dihydrosphingosine, and ceramides. Alternatively, Jurkat cells might tolerate a limiting value for each metabolite that is reached by either overexpression of SMS1 or photodamage.

Overexpression of SMS1 is accompanied by elevated resting levels of not only sphingomyelins, but also other sphingolipids, with concomitant downregulation of resting SPT activity. However, following PDT, there was no effect on SPT activity in either empty-vector transfectants or SMS1-overexpressors. Similarly, we have shown no effect of PDT on SPT activity in Jurkat parental cells [5]. Besides reduced inhibition of SMS after PDT, overexpression of SMS1 is associated with upregulation of GCS at the high PDT dose. Similarly, increased GCS activity has been reported in chemoresistant leukemia cells [6]. The data suggest that the observed changes in sphingolipid levels at rest and post-PDT are affected not only by overexpression of SMS1 but also by subsequent alterations in the activities of SPT and GCS.

We have shown pharmacologic and genetic evidence supporting a role of *de novo* sphingolipids in apoptosis after photodamage [3–5,15]. Here we show that SMS1-overexpressors are not only defective in ceramide response but also resistant to apoptosis after PDT. Unlike a recent report from Van Blitterswijk and colleagues [22], other studies have shown a correlation between apoptotic resistance and increased SMS activity [6,23]. Although it has been suggested that SMS1 is primarily responsible for production of sphingomyelin [24], our data support the hypothesis that SMS1 is a factor affecting both ceramide and apoptotic responsiveness of cells to photodamage. The Golgi membrane-SMS1 isoform is a more likely PDT target than the predominantly plasma membrane SMS2 isoform, since Pc 4 localizes in the inner cellular membranes and not in the plasma membrane [25], and the initial photodamage is expected to be confined to the former, e.g., Golgi membrane. Furthermore, in SMS1-overexpressors at the high PDT dose, GCS, another Golgi membrane protein [26], was upregulated, which might contribute to a greater resistance to apoptosis at that dose compared to other two PDT doses, at which there was no upregulation of GCS. Similarly, a correlation between increased GCS activity and chemoresistance has been observed [6]. Hence, our findings imply that SMS1 and GCS are PDT molecular targets with a potential of regulating apoptosis.

Acknowledgments

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