De Novo Ceramide Accumulation Due to Inhibition of Its Conversion to Complex Sphingolipids in Apoptotic Photosensitized Cells*

The oxidative stress induced by photodynamic therapy (PDT) with the photosensitizer phthalocyanine 4 is accompanied by increases in ceramide mass. To assess the regulation of de novo sphingolipid metabolism during PDT-induced apoptosis, Jurkat human T lymphoma and Chinese hamster ovary cells were labeled with [14C]serine, a substrate of serine palmitoyltransferase (SPT), the enzyme catalyzing the initial step in the sphingolipid biosynthesis. A substantial elevation in [14C]ceramide with a concomitant decrease in [14C] sphingomyelin was detected. The labeling of [14C]ceramide was completely abrogated by the SPT inhibitor ISP-1. In addition, ISP-1 partly suppressed PDT-induced apoptosis. Pulse-chase experiments showed that the contribution of sphingomyelin degradation to PDT-initiated increase in de novo ceramide was absent or minor. PDT had no effect on either mRNA amounts of the SPT subunits LCB1 and LCB2, LCB1 protein expression, or SPT activity in Jurkat cells. Moreover in Chinese hamster ovary cells LCB1 protein underwent substantial photodestruction, and SPT activity was profoundly inhibited after treatment. We next examined whether PDT affects conversion of ceramide to complex sphingolipids. Sphingomyelin synthase, as well as glucosylceramide synthase, have been implicated in ceramide generation during oxidative stress-de novo. Here we provide the first evidence from Pc 4-photosensitized Jurkat and CHO cells that (i) in the absence of SPT up-regulation PDT induces accumulation of de novo ceramide by inhibiting its conversion to complex sphingolipids. Ceramide is a result of inhibition of its conversion to complex sphingolipids. This paper is available online at http://www.jbc.org

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The oxidative stress induced by photodynamic therapy (PDT) with the photosensitizer phthalocyanine 4 is accompanied by increases in ceramide mass. To assess the regulation of de novo sphingolipid metabolism during PDT-induced apoptosis, Jurkat human T lymphoma and Chinese hamster ovary cells were labeled with [14C]serine, a substrate of serine palmitoyltransferase (SPT), the enzyme catalyzing the initial step in the sphingolipid biosynthesis. A substantial elevation in [14C]ceramide with a concomitant decrease in [14C] sphingomyelin was detected. The labeling of [14C]ceramide was completely abrogated by the SPT inhibitor ISP-1. In addition, ISP-1 partly suppressed PDT-induced apoptosis. Pulse-chase experiments showed that the contribution of sphingomyelin degradation to PDT-initiated increase in de novo ceramide was absent or minor. PDT had no effect on either mRNA amounts of the SPT subunits LCB1 and LCB2, LCB1 protein expression, or SPT activity in Jurkat cells. Moreover in Chinese hamster ovary cells LCB1 protein underwent substantial photodestruction, and SPT activity was profoundly inhibited after treatment. We next examined whether PDT affects conversion of ceramide to complex sphingolipids. Sphingomyelin synthase, as well as glucosylceramide synthase, have been implicated in ceramide generation during oxidative stress-de novo. Here we provide the first evidence from Pc 4-photosensitized Jurkat and CHO cells that (i) in the absence of SPT up-regulation PDT induces accumulation of de novo ceramide by inhibiting its conversion to complex sphingolipids.

Sphingolipids are widely present in eukaryotic cells. Their biosynthesis begins with the condensation of l-serine with palmitoyl-CoA to give rise to 3-ketosphinganine. The reaction is catalyzed by serine palmitoyltransferase (SPT)1 (1). At least two genes, LCB1 and LCB2, encoding two respective SPT subunits, are essential for expression of mammalian SPT activity (1). 3-Ketosphinganine is reduced to form sphinganine (dihydro sphingosine). In mammalian cells sphinganine is acylated to produce dihydroceramide, which is desaturated to give rise to ceramide (2). In the reactions catalyzed by phosphatidylcholine:ceramide phosphocholine transferase (sphingomyelin synthase) and UDP-glucosceramide glucosyltransferase (glucosylceramide synthase) ceramide is converted to sphingomyelin and glucosylceramide, respectively (3). Glucosylceramide is a building block for more complex glycosphingolipids (4). Although ceramide is the predominant hydrophobic backbone for numerous complex sphingolipids, some sphingolipids are derived from dihydroceramide and phytoceramide (3).

Ceramide has been suggested to mediate various biological processes (3, 5, 6). De novo ceramide has been associated with apoptotic cell death (7–12). SPT, as well as sphingomyelin synthase and glucosylceramide synthase, have been implicated in cytotoxic responses (4, 13). However, the details of the regulation of de novo ceramide generation during oxidative stress-induced apoptosis are unclear.

The aim of the present study was to address de novo ceramide production in apoptosis after the novel oxidative stress inducer photodynamic therapy (PDT). In PDT, after the uptake of a photosensitive dye, the dye is activated by red light with subsequent formation of reactive oxygen species to induce cell death (14). Apoptosis is an important mechanism in tumor ablation by PDT (15). We have demonstrated in a variety of cell types that apoptosis induced by photosensitization with the silicon phthalocyanine 4 (Pc 4) is accompanied by increases in ceramide mass (16–22), as well as sphinganine (23). We have also shown that de novo sphingolipids are involved in initiation of mitochondrial as well as extramitochondrial apoptosis after Pc 4-PDT (23, 24).

Here we provide the first evidence from Pc 4-photosensitized Jurkat and CHO cells that (i) in the absence of SPT up-regulation accumulation of de novo ceramide is a result of inhibition of its conversion to complex sphingolipids and that (ii) de novo ceramide may play an important role in PDT-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—The phthalocyanine photosensitizer Pc 4, HOSiPcOsi-(CH2)n1(CH2)n2N(CH3)2, was supplied by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). ISP-1 (Myriocin) and DEVD-7-amino-4-methyl-coumarin were from Biomol. Ceramide, glucocerebrosides, sphinganine, and sphingomyelin were from Matreya. C14-NBD-ceramide, phosphatidylethanolamine, and phosphatidylserine were from Avanti. t-12,14-Cerine (5.22 GBq/mmol) and t-3,5-[3H]Spermidine (962 GBq/mmol) were from Amersham Biosciences. Hoehst dye 33342 was from Molecular Probes. Basic media and sera were...
from In vitrogen and Hydacyte, respectively. Mouse monoclonal anti-LCBI and anti-KDEL (1:200) was antibodies in BD Bioscience and Stressgen, respectively. Anti-rabbit IgG (Bio-Rad) and anti-mouse IgG (Amersham Biosciences) horseradish peroxidase-labeled secondary antibodies were used. Thin layer chromatography (TLC) plates (aluminum sheets of silica gel 60) were from EM Science.

Cell Culture and Treatments—Jurkat human lymphoma T cells were purchased from American Type Culture Collection. Jurkat cells were cultured in RPMI 1640 medium (supplemented with 10% fetal bovine serum). CHO cells were grown in Ham’s F-12 medium (supplemented with 10% newborn calf serum). All cell cultures were grown in medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and were maintained at 37 °C in a 5% CO₂ atmosphere. For experiments, an aliquot of a stock solution of Pe (0.5 mM in dimethyl formamide) was added to the cells in the culture medium to give a final concentration of 200 and 500 mM for Jurkat and CHO cells, respectively. After overnight incubation, the cells were irradiated with red light (2 milliwatts/cm², λmax = 670 nm) using a light-emitting diode array light source (EOFOS) at various fluences at room temperature. Following PDT, cells were incubated at 37 °C for desired periods of time. CHO cells were harvested using trypsin (0.25%) and gentle scraping in modified Hank’s (supplemented with 1.2 mM EDTA and 25 mM HEPEs, pH 7.4).

Metabolic Radiolabeling of Cellular Lipids—In pulse experiments, following PDT treatments in the culture medium were labeled with [14C]serine (28 kBq) for 2 h. In some experiments, ISP-1 (1 μM) was added 1 h prior to PDT. In pulse-chase experiments Pe 4-treated cells were labeled with [14C]serine (28 kBq) for 2 h, incubated with 10 mM t-serine for 30 min, irradiated, and then incubated for the desired periods of time. Following post-treatment incubations, cells were harvested by centrifugation at 4 °C. Total extracted cellular lipids (25) were separated by TLC (mixture of propyl alcohol, chloroform, methanol, 0.25% potassium chloride; 25:25:25:10:9, v/v). After chromatography, the TLC plates were exposed to PhosphorImager screens (Amersham Biosciences) for 48 h. The samples were analyzed by a STORM 860 (Amersham Biosciences) imaging system. Data were normalized per milli-curies of [14C]serine (28 kBq).

TLC Detection of Intracellular [14C]Serine—Untreated or PDT-treated cells in the culture medium were labeled with [14C]serine (28 kBq) for desired periods of time. After incubations, cells were harvested rapidly on ice and washed three times with ice-cold PBS to remove exogenous [14C]serine. After extraction (25), aqueous upper phases were dried down and separated by TLC (0.6% NaCl, methanol, 28% ammonia, 25:10:1, v/v) as described elsewhere (27). After chromatography, the TLC plates were exposed to PhosphorImager screens (Amersham Biosciences) for 24 h. The samples were analyzed by the STORM 860 (Amersham Biosciences) imaging system. Serine and phosphoserine bands were identified by comparison to comittanly run standard curve comprised of known amounts of lipids. The Rv values for ceramide, glucosylceramide, and sphingomyelin were 0.83, 0.65, and 0.04, respectively. The Rv values for phosphatidyl ethanolamine and phosphatidyserine were 0.28 and 0.14, respectively.

Reverse Transcription—Total RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) according to the manufacturer’s instructions. Following RNA purification, DNase treatment (Ambion) was performed following the manufacturer’s directions to ensure that there was no contaminating genomic DNA.

RNA Isolation—Following PDT treatment, Jurkat cells (5 × 10⁶) were harvested and washed in PBS. Total cellular RNA was isolated from cell lysates using an RNAeasy minikit (Qiagen) according to the manufacturer’s instructions. Following RNA purification, DNase treatment (Ambion) was performed following the manufacturer’s directions to ensure that there was no contaminating genomic DNA.

SPT Activity Assay—The enzyme activity was assayed as described previously (34). Enzyme activity in 100 μg of microsomal membranes was measured in 50 mM HEPES (pH 7.4), 5 mM MgCl₂, 5 mM EDTA, and 50 μM pyridoxal 5’-phosphate. The reaction was initiated by the addition of 200 μM palmitoyl-CoA and 740 kBq of [1-14C]serine (1 mCi, final concentration). A control containing all of the components except palmitoyl-CoA was included as well. Following incubation for 10
min at 37 °C, the reaction was terminated with 0.2 ml of 0.5 N \( \text{NH}_4 \text{OH} \). The \( \text{H} \)-labeled lipid product 3-ketosphinganine was extracted, and the radioactivity was measured by scintillation counting. SPT activity was expressed as pmol of 3-ketosphinganine generated/10 min/mg of protein after subtracting the background radioactivity (i.e. the minus palmitoyl-CoA control).

**Sphingomyelin and Glucosylceramide Synthase Activity Assays**—The sphingomyelin synthase activity assay was performed as described previously (35). Enzyme activity in 50–100 µg of microsomal membranes was measured in 50 mM HEPES (pH 7.5), 5 mM EDTA, and 10 µM C\(_6\)-NBD-ceramide complexed with fatty acid-free bovine serum albumin (0.1 mM). To determine glucosylceramide synthase activity, 500 nM UDP-glucose was included in the assay mixture (35). For the incubation for 5 min at 37 °C, the reaction was terminated with chloroform/methanol (1:2, v/v). C\(_6\)-NBD-ceramide-labeled lipid products were extracted and separated by TLC using chloroform/methanol/water (65:25:4, v/v), and their fluorescence was detected and quantified by the STORM 860 imaging system. Sphingomyelin synthase and glucosylceramide synthase activities were expressed as pmol of sphingomyelin and glucosylceramide, respectively, generated/5 min/mg of protein after subtracting the background fluorescence.

**DEVDase Activity**—The enzyme activity was determined as described previously (36). Approximately 1–2 \( \times \) 10\(^5\) cells were collected by centrifugation and washed once with PBS. The cells were resuspended in 100 µl of a lysis buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 µM pepstatin, 100 µM leupeptin, and 0.5% Triton X-100), incubated on ice for 20 min, and then sonicated on ice with a 20-s burst (two times) using the VirSonic 100 (Virtis) sonicator and stored at −140 °C until use. Aliquots (50 µg of protein) were incubated at 37 °C for 1 h in 60 µl of caspase reaction buffer (25 mM HEPES, pH 7.4, 1.5 mM MgCl\(_2\), 10% sucrose, 0.1% CHAPS, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 µM pepstatin, 100 µM leupeptin, 100 µM DEVD-7-amino-4-methyl-coumarin). The released fluorescence of the cleaved DEVD substrate was measured in an F-2500 Hitachi spectrofluorometer (380 nm excitation and 460 nm emission).

**Hoechst Staining**—Nuclear apoptotic changes were determined by staining with the DNA-binding Hoechst 33342 dye as described previously (37). After a 30-min fixation at room temperature with formalin, the cell pellets were washed with PBS and stained with Hoechst dye (24 µg/ml, overnight at 4 °C), and 200–300 cells were counted for the incidence of apoptotic chromatin condensation under a fluorescence microscope (Zeiss).

**Statistical Analysis**—Results were expressed as mean ± S.E. Statistical analyses were performed by Student’s \( t \) test. Significance was defined as a two-tailed \( p \) \(< 0.05.

**RESULTS**

**PDT-induced Increased Labeling of Ceramide with \(^{14}\)C[Serine Is ISP-1-sensitive in Jurkat Cells**—To assess the effect of PDT on the de novo sphingolipid biosynthesis in cells, \(^{14}\)C[serine was used for labeling of cellular lipids. A dose-dependent increase in \(^{14}\)C[seride was observed at 2 h after PDT (Fig. 1, A and C). Specifically exposure of Pc 4 (200 nM)-treated Jurkat cells to light fluences of 135 or 270 mJ/cm\(^2\) did not affect intracellular \(^{14}\)C[serine amounts between control and PDT-treated samples at any time point. We also tested the effect of different PDT doses on intracellular \(^{14}\)C[serine accumulation at 2 h. Exposure of Pc 4 (200 nM)-treated Jurkat cells to light fluences of 135 or 270 mJ/cm\(^2\) did not affect substantially intracellular \(^{14}\)C[serine amounts (Fig. 3B). Only at the highest PDT dose (200 nM Pc 4 + 400 mJ/cm\(^2\)) was some loss of \(^{14}\)C[serine observed (Fig. 3B). Overall PDT did not affect intracellular \(^{14}\)C[serine accumulation under the conditions used for metabolic labeling. Thus, it is unlikely that PDT-induced changes in the amounts of \(^{14}\)C[serine-labeled lipids are due to potential PDT effects on serine uptake during metabolic labeling.

**SPT Is Not Up-regulated after PDT in Jurkat Cells**—We have shown that sphinganine generation after PDT is ISP-1-sensitive (23). Since SPT can be up-regulated by inducers, such as UV radiation (39) or endotoxin (40), and photo-oxidative stress can up-regulate certain genes (41–45), we first tested whether PDT can induce up-regulation of mRNA for SPT subunits LCB1 and LCB2. Based on the findings that rapid PDT apoptosis is not associated with either transcription or new protein synthesis (46), we hypothesized that during delayed rather than rapid apoptosis SPT mRNA may be up-regulated. Exposure of Jurkat cells to various PDT doses (200 nM Pc 4 + 135, 270, or 400 mJ/cm\(^2\)) leads to apoptosis at 1.5 h in 17, 40, and 46% cells, respectively (23). The same PDT doses did not up-regulate mRNA of either LCB1 or LCB2. Based on the findings that rapid PDT apoptosis is not associated with either transcription or new protein synthesis (46), we hypothesized that during delayed rather than rapid apoptosis SPT mRNA may be up-regulated. Exposure of Jurkat cells to various PDT doses (200 nM Pc 4 + 135, 270, or 400 mJ/cm\(^2\)) leads to apoptosis at 1.5 h in 17, 40, and 46% cells, respectively (23). The same PDT doses did not up-regulate mRNA of either LCB1 or LCB2 (Table I). At the lower PDT doses (200 nM Pc 4 + 20 or 40 mJ/cm\(^2\)), apoptosis was not observed until 24 h after PDT (not shown). The low PDT doses did not up-regulate LCB1 or LCB2 transcript up to 24 h (not shown). These results demonstrate the absence of SPT mRNA up-regulation after Pc 4-PDT irrespective of the rapidity of apoptosis.

The possibility of SPT up-regulation was further tested at the protein level. Pc 4-treated Jurkat cells were exposed to different light fluences (135, 270, or 400 mJ/cm\(^2\)), and somonial LCB1 protein amounts were analyzed by Western blot. As depicted in Fig. 4A, there was no change in LCB1 protein expression. Similarly LCB1 protein levels remained unchanged up to 16 h post-PDT (200 nM Pc 4 + 135 mJ/cm\(^2\); Fig. 4B).

Since SPT can be activated without transcriptional up-regulation and increased protein expression (8, 10, 47), in the next
series of experiments we measured SPT activity in microsomes isolated from either control or PDT-treated Jurkat cells. The enzyme activity did not change when cells were exposed to various PDT doses (200 nM Pc 4, 135, 270, or 400 mJ/cm²; Fig. 4C). Similarly SPT activity was not affected by PDT (200 nM Pc 4 + 135 mJ/cm²) up to 16 h (Fig. 4D). At 24 h SPT activity was attenuated by 23% (p < 0.05) compared with Pc 4-treated samples. Since at that time point ~90% of Jurkat cells were apoptotic (not shown), the inactivation of SPT is probably a consequence of cell death. Overall our data support no upregulation of SPT by PDT.

Sphingomyelin Synthase and Glucosylceramide Synthase Are Inhibited after PDT in Jurkat Cells—Since [14C]serine-labeled ceramide amounts are elevated in the absence of SPT activation, we tested whether PDT affects the conversion of de novo ceramide to complex sphingolipids. Specifically the activities of sphingomyelin synthase and glucosylceramide synthase post-PDT were determined since both enzymes can be affected in response to different stimuli (48–50). As depicted in Fig. 5A, sphingomyelin synthase was profoundly inhibited in Jurkat cells. PDT doses (200 nM Pc 4 + 135, 270, or 400 mJ/cm²) inhibited the enzyme activity at 1 h by 85, 89, and 94%, respec-
The time course experiments showed that PDT (200 nM Pc4/1100135 mJ/cm²)-induced inactivation of sphingomyelin synthase persisted for at least 16 h (Fig. 5B).

Glucosylceramide synthase was also inhibited in Pc4/110060 min were 2.1/110060.0 and 6.3/110060.5, respectively. Cer, ceramide; Con, untreated control; Pc4, Pc4-treated control; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

**TABLE I**
Reverse transcription-PCR of hLCB1 and hLCB2 RNA in Jurkat cells post-PDT

| PDT          | hLCB1     | hLCB2     |
|--------------|-----------|-----------|
|              | 2 h       | 4 h       | 2 h       | 4 h post-PDT |
| Light fluence|           |           |           |              |
| 135 mJ/cm²   | 1.2 ± 0.2 | 1.4 ± 0.2 | 1.3 ± 0.1 | 0.1 ± 1.2    |
| 270 mJ/cm²   | 0.1 ± 1.5 | 1.3 ± 0.1 | 0.1 ± 1.2 | 0.1 ± 1.1    |
| 400 mJ/cm²   | -1.4 ± 0.3| 1.5 ± 0.2 | -2.1 ± 0.7| -0.2 ± 1.3   |
| Pc4          | -1.2 ± 0.1|           | 1.1 ± 0.1 |              |

60 min were 2.1 ± 0.0 and 6.3 ± 0.5, respectively. Cer, ceramide; Con, untreated control; Pc4, Pc4-treated control; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

**FIG. 3.** Effect of PDT on [14C]serine uptake in Jurkat cells. A and B, after overnight preincubation with Pc4 (200 nM), Jurkat cells were irradiated at 270 mJ/cm² (A) or at the indicated light fluences (B). Cells were exposed to [14C]serine immediately after PDT. At the indicated times (A) or at 2 h (B), cells were rapidly harvested, washed, and processed for TLC analysis and phosphorimaging as described under “Experimental Procedures.” A, the data (pixels/mg of protein) are shown as means ± S.E. of four to five independent determinations. B, representative results from three to five independent determinations are shown. Con, untreated control; Pc4, Pc4-treated control; P-Serine, phosphoserine.

**FIG. 2.** Pulse-chase labeling of lipids with [14C]serine in Jurkat cells after PDT. After overnight preincubation with Pc4 (200 nM), Jurkat cells were exposed to [14C]serine for 2 h, treated with L-serine (10 mM) for 30 min, irradiated at 270 mJ/cm², and then incubated for the indicated periods of time. After incubation, cells were processed, and 14C-labeled lipids were analyzed by TLC and phosphorimaging as described under “Experimental Procedures.” A, representative TLC results from four to five independent determinations are shown. B and C, the data are expressed as -fold change relative to time-matched untreated controls. The plots for respective lipids are shown in the following order: B, [14C]ceramide; C, [14C]sphingomyelin. The data are shown as means ± S.E. of four to five independent determinations. The resting Pc4-treated control values (pixels/mg of protein) for ceramide and sphingomyelin at
incubated for 1 h.

After overnight preincubation with Pc 4 (200 nM), Jurkat cells were either irradiated at the indicated light fluences and then incubated for 1 h (A) or irradiated at 135 mJ/cm² and then incubated for the indicated periods of time (B and D).

**Effects of PDT on LCB1 expression and SPT activity in Jurkat cells.** After overnight preincubation with Pc 4 (200 nM), Jurkat cells were either irradiated at the indicated light fluences and then incubated for 1 h (A and C) or irradiated at 135 mJ/cm² and then incubated for the indicated periods of time (B and D). After harvesting the cells, microsomes were isolated. A and B, microsomal proteins were separated by 10% SDS-PAGE and analyzed by Western blotting with anti-LCB1 or anti-KDEL. Representative results from at least two independent experiments are shown. C and D, microsomal SPT activity was measured as described under “Experimental Procedures.” The data are expressed as pmol/mg of protein/10 min and are shown as means ± S.E. of three to 10 independent determinations.

**Experimental Procedures.**

To test whether the observations obtained from Jurkat cells are reproducible in other cell types, we examined the effects of PDT on metabolic labeling of ceramide in CHO cells. PDT doses (500 nM Pc 4 + 100 or 200 mJ/cm²) increased [14C]labeling of ceramide at 2 h by 2.7- and 8.4-fold, respectively (Fig. 6, A and C). The effect was abolished when cells were co-exposed to PDT + ISP-1 (Fig. 6, B and C). ISP-1 alone also completely inhibited incorporation of [14C]serine into ceramide at rest (Fig. 6, B and C). Hence, in CHO cells, similar to Jurkat cells, PDT initiates substantial increases in ISP-1-sensitive [14C]ceramide.

Increases in [14C]ceramide were paralleled by a dose-dependent inhibition of the [14C]serine incorporation into sphingomyelin post-PDT (Fig. 6, A and D). PDT + ISP-1 abolished the incorporation of [14C]serine into sphingomyelin (Fig. 6, B and D). ISP-1 alone also abrogated labeling of sphingomyelin with [14C]serine at rest (Fig. 6, B and D).

PDT (500 nM Pc 4 + 100 and 200 mJ/cm²) increased labeling of glucosylceramide with [14C]serine by 2.0 and 2.7-fold, respectively (Fig. 6, A and E). PDT + ISP-1 completely inhibited the incorporation of [14C]serine into glucosylceramide (Fig. 6, B and E). ISP-1 alone also abolished basal glucosylceramide labeling (Fig. 6, B and E).

The incorporation of [14C]serine into phosphatidyserine and phosphatidylethanolamine was inhibited after PDT (Fig. 6A and B). Neither ISP-1 alone nor PDT + ISP-1 had any effect on [14C]labeling of phosphatidyserine (Fig. 6B). While resting [14C]phosphatidylethanolamine was unaffected by ISP-1, PDT + ISP-1 inhibited [14C]labeling of phosphatidylethanolamine (Fig. 6B). Because sphingoid bases, like sphinganine that is produced in the de novo sphingolipid biosynthesis, can be catabolized to phosphoethanolamine, which is then used for biosynthesis of phosphatidylethanolamine (51), this may explain why PDT + ISP-1 further inhibits [14C]labeling of phosphatidylethanolamine.

**Effects of PDT on Catabolism of Complex Sphingolipids and [14C]Serine Uptake in CHO Cells**—To test whether catabolism of sphingolipids is affected by PDT in CHO cells, pulse-chase experiments were carried out. While no significant changes in either [14C]ceramide or glucosylceramide were detected (Fig. 7, A, B, and D), [14C]labeling of sphingomyelin was attenuated (Fig. 7C). Specifically, following PDT [14C]sphingomyelin amounts were reduced by 35, 40, and 43% at 5, 15, and 60 min, respectively. These findings suggest that PDT-induced decreases in [14C]ceramide in CHO cells is, at least in part, due to the degradation of sphingomyelin. In addition, [14C]phosphatidylethanolamine and [14C]phosphatidyserine amounts were also inhibited post-PDT (Fig. 7A), indicating a rapid turnover of the two lipids.

The effect of PDT on the [14C]serine pool was also assessed in CHO cells. Exposure of Pc 4 (500 nM)-treated CHO cells to light fluences of 100 or 200 mJ/cm² did not substantially affect intracellular [14C]serine amounts (Fig. 7E). However, a higher PDT dose (500 nM Pc 4 + 400 mJ/cm²) induced substantial loss of [14C]serine (not shown). There was no difference in cellular [14C]serine between untreated and PDT (500 nM Pc 4 + 200 mJ/cm²)-treated CHO cells at either 0 or 120 min (not shown). The data support that PDT (500 nM Pc 4 + 100 or 200 mJ/cm²) had no effect on [14C]serine uptake in CHO cells.

**FIG. 4. Effects of PDT on LCB1 expression and SPT activity in Jurkat cells.** After overnight preincubation with Pc 4 (200 nM), Jurkat cells were either irradiated at the indicated light fluences and then incubated for 1 h (A and C) or irradiated at 135 mJ/cm² and then incubated for the indicated periods of time (B and D). After harvesting the cells, microsomes were isolated. A and B, microsomal proteins were separated by 10% SDS-PAGE and analyzed by Western blotting with anti-LCB1 or anti-KDEL. Representative results from at least two independent experiments are shown. C and D, microsomal SPT activity was measured as described under “Experimental Procedures.” The data are expressed as pmol/mg of protein/10 min and are shown as means ± S.E. of three to 10 independent determinations. D, the zero point (155 ± 16 pmol/mg of protein/10 min) represents untreated controls, which were similar to Pc 4-treated controls (161 ± 14 pmol/mg of protein/10 min). Con, untreated control; Pc 4, Pc 4-treated control.
Sphingomyelin Synthase and Glucosylceramide Synthase Are Inhibited after PDT in CHO Cells—

We next tested whether PDT affects the activities of sphingomyelin synthase and glucosylceramide synthase in CHO cells. Similar to our findings in Jurkat cells, PDT profoundly inhibited sphingomyelin synthase in CHO cells (Fig. 8A). PDT doses (200 nM Pc4/H11001 100, 200, or 400 mJ/cm²) inhibited the enzyme activity at 1 h by 50, 84, and 92%, respectively. Glucosylceramide synthase was also inhibited in Pc4-photosensitized CHO cells. PDT doses (200 nM Pc4/H11001 100, 200, or 400 mJ/cm²) attenuated the enzyme activity at 1 h by 24, 60, and 81%, respectively (Fig. 8B). Therefore, both sphingomyelin synthase and glucosylceramide synthase were also inhibited in CHO cells post-PDT.

PDT Induces Loss of LCB1 in CHO Cells—

In the next series of studies we addressed the regulation of LCB1 by PDT directly. To determine the expression amounts of LCB1, Pc4 (500 nM)-treated CHO cells were exposed to various light fluences (100, 200, or 400 mJ/cm²) and then incubated for 1 h. Microsomal LCB1 protein amounts were analyzed and quantified by Western blot and chemiluminescence image analyzer. PDT doses reduced LCB1 protein amounts by 41, 63, and 90% (Fig. 9A). At the highest PDT dose (500 nM Pc4 + 400 mJ/cm²), the expression amount of LCB1 protein was reduced by ~90% immediately after PDT (Fig. 9B).

We next measured SPT activity in microsomes isolated from either control or PDT-treated CHO cells. PDT caused a dose-dependent inhibition of SPT at 1 h, since co-exposure of CHO
Fig. 6. Effect of PDT with or without ISP-1 on the incorporation of $[^{14}C]$serine into lipids in CHO cells. After overnight preincubation with Pc 4 (500 nM), CHO cells were irradiated at the indicated light fluences (A and C–E) or pretreated with ISP-1 (1 μM) for 1 h (B–E) prior to irradiation at 200 mJ/cm$^2$ (B). Cells were exposed to $[^{14}C]$serine immediately after PDT. After 2 h, cells were processed, and $[^{14}C]$-labeled lipids were analyzed by TLC and phosphorimaging as described under “Experimental Procedures.” A and B, representative results from two to six independent determinations are shown. C–E, the data are expressed as fold change relative to time-matched untreated controls. The plots of pulse labeling with $[^{14}C]$serine for respective lipids are shown in the following order: C, $[^{14}C]$ceramide; D, $[^{14}C]$sphingomyelin; E, $[^{14}C]$glucosylceramide. The data are shown as means ± S.E. of two to seven independent determinations. The resting untreated control values (pixels/mg of protein) for ceramide, sphingomyelin, and glucosylceramide were 35.6 ± 1.0, 145.7 ± 9.5, and 10.1 ± 1.0, respectively. Cer, ceramide; Con, untreated control; GlcCer, glucosylceramide; I, ISP-1; Pc 4, Pc 4-treated control; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.
Effects of PDT on catabolism of newly synthesized sphingolipids and [14C]serine uptake in CHO cells. A–D, pulse-chase labeling of lipids with [14C]serine in CHO cells after PDT. After overnight preincubation with Pc 4 (500 nM), CHO cells were exposed to [14C]serine for 2 h, treated with L-serine (10 mM) for 30 min, irradiated at 200 mJ/cm², and then incubated for the indicated periods of time. After incubation, cells were processed, and 14C-labeled lipids were analyzed by TLC and phosphorimaging as described under "Experimental Procedures." A, representative TLC results from three independent determinations are shown. B–D, the data are expressed as -fold change relative to untreated controls. The plots for respective lipids are shown in the following order: B, [14C]ceramide; C, [14C]sphingomyelin; D, [14C]glucosylceramide. The data are shown as means ± S.E. of two to three independent determinations. The resting Pc 4-treated control values (pixels/mg of protein) at 60 min for ceramide, sphingomyelin, and glucosylceramide were 5.4 ± 0.5, 69.4 ± 9.8, and 4.0 ± 0.2, respectively. E, effect of PDT on [14C]serine
cells to Pc 4 (500 nM) and the light fluences of 100, 200, or 400 mJ/cm² reduced the activity of the enzyme by 23, 48, and 77%, respectively (Fig. 9C). The inhibition of the enzyme activity (74%) was detected as early as 0 min after PDT and was maintained for at least 1 h (Fig. 9D).

ISP-1 Inhibits DEVDase Activation and Apoptosis after Pc 4-PDT in CHO Cells—We have already demonstrated that ISP-1-induced abrogation of PDT-induced sphinganine generation amounts in CHO cells. After overnight preincubation with Pc 4 (500 nM), CHO cells were irradiated at the indicated light fluences and then incubated for 1 h. After the cells were harvested, microsomes were isolated, and the activities of the two enzymes were assayed. A, sphingomyelin synthase; B, glucosylceramide synthase. The data are expressed as -fold change relative to time-matched untreated controls and are shown as means ± S.E. of three to four independent determinations. The resting sphingomyelin synthase and glucosylceramide synthase activities were 190 ± 20 and 870 ± 130 pmol/mg of protein/5 min, respectively. Pc 4, Pc 4-treated control.

FIG. 8. Effects of PDT on sphingomyelin synthase and glucosylceramide synthase activities in CHO cells. In all experiments cells were treated overnight with Pc 4 (500 nM), irradiated at the indicated light fluences, and then incubated for 1 h. After the cells were harvested, microsomes were isolated, and the activities of the two enzymes were assayed. A, sphingomyelin synthase; B, glucosylceramide synthase. The data are expressed as -fold change relative to time-matched untreated controls and are shown as means ± S.E. of three to four independent determinations. The resting sphingomyelin synthase and glucosylceramide synthase activities were 190 ± 20 and 870 ± 130 pmol/mg of protein/5 min, respectively. Pc 4, Pc 4-treated control.

FIG. 9. Effects of PDT on LCB1 protein and SPT activity in CHO cells. After overnight preincubation with Pc 4 (500 nM), CHO cells were either irradiated at the indicated light fluences and then incubated for 1 h (A and C) or irradiated at 400 mJ/cm² and then incubated for 0 min (B) or the indicated periods of time (D). After harvesting the cells, microsomes were isolated. A and B, microsomal proteins were separated by 10% SDS-PAGE and analyzed by Western blotting with anti-LCB1 or anti-KDEL. Representative results from two independent experiments are shown. C and D, microsomal SPT activity was measured as described under “Experimental Procedures.” The data are expressed as pmol/mg of protein/10 min and are shown as means ± S.E. of three to 14 independent determinations. D, the zero point (477 ± 33 pmol/mg of protein/10 min) represents untreated controls, which were similar to Pc 4-treated controls (462 ± 32 pmol/mg of protein/10 min). Con, untreated control; Pc 4, Pc 4-treated control.

ISP-1 Inhibits DEVDase Activation and Apoptosis after Pc 4-PDT in CHO Cells—We have already demonstrated that ISP-1-induced abrogation of PDT-induced sphinganine generation amounts in CHO cells. After overnight preincubation with Pc 4 (500 nM), CHO cells were irradiated at the indicated light fluences. Cells were exposed to [14C]serine immediately after PDT. After 2 h, cells were rapidly washed and processed for TLC analysis and phosphorimaging as described under “Experimental Procedures.” Representative results from three independent determinations are shown. Cer, ceramide; Con, untreated control; GlcCer, glucosylceramide; Pc 4, Pc 4-treated control; PE, phosphatidylethanolamine; PS, phosphatidylserine; P-Serine, phosphoserine; SM, sphingomyelin.
the induction of apoptosis in response to the same PDT doses from 10.1, 19.6, and 30.2% to 3.9, 9.3, and 19.2%, respectively (Fig. 10B). Thus, there is a correlation between the inhibition of [14C]ceramide and apoptosis by ISP-1, suggesting the involvement of the de novo ceramide in the process.

DISCUSSION

This is the first report showing that in the absence of SPT up-regulation PDT initiates de novo ceramide generation via inhibition of its conversion to complex sphingolipids. PDT-induced increases in [14C]ceramide reflect de novo ceramide accumulation, since (i) the incorporation of the SPT substrate [14C]serine into ceramide was stimulated post-PDT, (ii) the SPT inhibitor ISP-1 abolished labeling of ceramide with [14C]serine, and (iii) PDT did not accelerate degradation of de novo synthesized complex sphingolipids in Jurkat cells (although accelerated degradation might occur in CHO cells). In addition, the pulse-chase data showed that [14C]ceramide amounts were not changed following PDT, ruling out the possibility that the increases in the amount of [14C]ceramide resulted from slower degradation of de novo synthesized ceramide in PDT-treated cells. The present data are consistent with the notion that previously observed PDT-induced increases in ceramide mass in numerous cell lines (16–22), including CHO (17) and Jurkat cells, are a result of de novo ceramide accumulation. Treatment of cells with ISP-1 suppresses not only PDT-induced accumulation of de novo ceramide but also PDT-induced apoptosis (Fig. 10 and Ref. 23). Hence, we propose that the lack of ceramide accumulation may cause apoptotic resistance to PDT. Collectively our findings further support the role of ceramide in apoptosis of photosensitized cells.

The following evidence from both Jurkat and CHO cells is consistent with sphingomyelin synthase as a molecular PDT target. (i) The enzyme was inactivated in a dose-dependent manner after treatment. (ii) [14C]Serine pulse labeling of sphingomyelin was inhibited post-PDT. Similarly, tumor necrosis factor and ceramide inhibit sphingomyelin synthase (48). Our findings strongly support that sphingomyelin synthase controls cellular ceramide amounts and functions, as suggested by Hannun and co-workers (52).

Glucosylceramide synthase has been shown to be activated (49) as well as inhibited (50) in response to some stimuli. However, the enzyme is not involved in apoptosis after etoposide and ionizing radiation in Jurkat cells (53). We have shown that in both Jurkat and CHO cells PDT-induced inhibition of glucosylceramide synthase was not as effective as the inactivation of sphingomyelin synthase (e.g. in Fig. 5 compare A and B with C and D). Despite glucosylceramide synthase inactivation by PDT, [14C]labeling of glucosylceramide increased in photosensitized CHO cells. The pulse-chase data showed no significant changes in [14C]glucosylceramide amounts, suggesting the absence of PDT-induced up-regulation of glucosylceramide catabolism. Perhaps accumulated de novo ceramide due to inhibition of sphingomyelin synthase can translocate to the site of glucosylceramide synthesis to increase [14C]-labeled glucosylceramide amounts despite partial inhibition of glucosylceramide synthase.

The biochemical evidence from CHO cells supports that LCB1 is a Pc 4-PDT target, since (i) the native 52-kDa LCB1 protein is lost on Western blots, and (ii) the SPT activity is inhibited. Both effects are dose-dependent. The observations that the inactivation and the disappearance of LCB1 are immediate (0 min post-PDT) indicate that PDT induces direct photodamage of the LCB1 subunit of SPT. Rapid photodamage is predicted, since the major PDT damaging reactive oxygen

\[ \text{\textsuperscript{[3]}D. Separovic, unpublished observations.}\]
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species, singlet oxygen, which is formed where the photosensitizer is localized, has a short lifetime (<0.04 μs) and a short radius of action (<0.02 μm) (54). Hence it is expected that most of the singlet oxygen will react very near to its site of production. PC 4 is localized to intracellular membranes, including the endoplasmic reticulum (55), where SPT is localized as well (32). Of note, Bel-2, which can also be found in the endoplasmic reticulum, is directly damaged by PC 4-PDT (56, 57). Moreover, we have shown that PDT affects metabolic labeling of phosphatidylserine, indicating that PDT also damages phosphatidylserine syntheses, which are localized to the endoplasmic reticulum (58). In contrast to CHO cells, in Jurkat cells SPT is up-regulated, though the reasons for the differential susceptibility of SPT in Jurkat cells (Fig. 7C versus Fig. 2C), suggesting that the effect of PDT on catabolism of sphingomyelin is also cell type-dependent.

In summary, our data strongly support that the observed increases in de novo ceramide in photosensitized apoptotic cells in the absence of SPT up-regulation are a result of inhibition of ceramide conversion to complex sphingolipids. Our novel findings imply that PDT-induced inhibition of conversion of ceramide to complex sphingolipids plays a critical role in regulating de novo ceramide amounts. Our data also indicate that sphingomyelin synthase and glucosylceramide synthase are key regulators of both de novo ceramide generation and its putative apoptotic function in photosensitized cells.

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De Novo Ceramide Accumulation Due to Inhibition of Its Conversion to Complex Sphingolipids in Apoptotic Photosensitized Cells
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