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Ceramide Generation in Response to Photodynamic Treatment of L5178Y Mouse Lymphoma Cells[1]

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ABSTRACT

Photodynamic therapy, a novel cancer treatment using a photosensitive dye and visible light, produces an oxidative stress in cells, often leading to apoptotic cell death. Because ceramide is a second messenger that has been associated with stress-induced apoptosis, we investigated a possible link between photodynamic treatment (PDT), ceramide, and apoptosis in L5178Y-R (LY-R) cells. The cells undergo rapid apoptosis, initiating within 30 min of PDT. After a dose of PDT producing a 99.9% loss of clonogenicity, LY-R cells responded by an increased production of ceramide, which reached a maximum level in 60 min. For a constant light fluence and varying concentrations of the phthalocyanine photosensitizer Pc 4 [HOSiPcOSi$(CH_3)_2(CH_2)_3N(CH_3)_2$], the ED_{50} for ceramide generation (46 nM) was similar to the LD_{50} for clonogenic cell death (40 nM). We suggest that the PDT-stimulated increase in synthesis of ceramide in LY-R cells may be coupled to PDT-induced apoptosis. When the cells were exposed to exogenous *N*-acetyl-sphingosine (10 μM), apoptotic changes were observed only after 12–24 h. The delayed apoptotic response to the synthetic ceramide may be due to an induction of apoptosis by a different route than the one used by PDT.

INTRODUCTION

Photodynamic therapy is a cancer treatment using a porphyrin-related photosensitizer that is activated by visible light. As a consequence of the sensitizer uptake by cells or tumors and photoactivation, singlet oxygen and other reactive oxygen species are generated, causing damage to cellular organelles and, ultimately, cell death and tumor ablation (1). PDT[3] has been shown to induce apoptosis *in vitro* (2–4) and *in vivo* (5–7). Apoptosis induction seems to depend on both the photosensitizer and cell line (2, 3, 8). In mouse lymphoma LY-R cells, the apoptotic response to PDT initiates rapidly, with extensive oligonucleosomal DNA fragmentation observed as early as 30 min post-PDT. The process does not require new protein or RNA synthesis and seems to involve the activation of phospholipases A_2 and C, as well as an increase in intracellular Ca^{2+} (9).

Because PDT photooxidizes cellular targets, the treatment is considered an oxidative stress. Cellular stressors, such as UV irradiation, hydrogen peroxide, or X-rays, cause ceramide generation (10–12), and elevated ceramide levels have been linked to stimulation of the SAPK/JNK signaling pathway and to the induction of apoptosis (12). Moreover, the SAPK cascade, as well as the p38/hyperosmotic glucose-activated kinase 1 protein kinase pathway, is activated in response to PDT in murine keratinocytes (13). Therefore, we asked whether PDT can cause increased synthesis of ceramide and whether ceramide production is linked to apoptosis in PDT-treated LY-R cells.

MATERIALS AND METHODS

Materials. The phthalocyanine photosensitizer Pc 4, HOSiPcOSi-$(CH_3)_2(CH_2)_3N(CH_3)_2$ (14), was supplied by Drs. Ying-syi Li and Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). Octylglucoside and DTT were obtained from Boehringer Mannheim (Indianapolis, IN); C2-ceramide, C2-dihydroceramide, and *Escherichia coli sn*-1,2-DAG kinase were from Calbiochem (La Jolla, CA), whereas ceramide (type III), cardiolipin, DETAPAC, EGTA, and $MgCl_2$ were from Sigma (St. Louis, MO). [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). All other chemicals were purchased from Fisher (Pittsburgh, PA). TLC plates (aluminum sheets of Silica Gel 60) were from EM Industries (Gibbstown, NJ).

Cell Culture and Treatments. Suspension cultures of mouse lymphoma LY-R cells were grown in Fisher's medium supplemented with 10% heat-inactivated horse serum and 5 mM glutamine in a 37°C incubator with humidified air containing 5% CO_2. The medium was changed 2–3 times a week. For experiments, cells were seeded at 1.3–2 × 10^5 cells/ml and grown overnight (13–19 h) in Fisher's medium containing 1% horse serum (LSM). Cell viability was assessed by trypan blue dye exclusion. Cell survival was determined by clonogenic assay in soft agar medium (15). U937 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured as described previously (12).

For PDT experiments, an aliquot of a stock solution of Pc 4 (0.5 mM in dimethyl formamide) was added to the cells in T-75 culture flasks containing 40 ml of LSM to give the desired concentrations (50–300 nM). After overnight incubation, the cells were collected by centrifugation, resuspended in 0.1 volume of LSM, transferred to 60-mm Petri dishes, and returned to the incubator for 15 min. Cultures were irradiated using an EFOS LED array (EFOS, Mississauga, Canada; λ_{max} ~670–675 nm; 1.5 mW/cm^2, 45 mJ/cm^2, 30 s) and then transferred to T-75 flasks containing 34 ml of gassed LSM and incubated for intervals of 10–120 min before harvest.

In the experiments in which cells were treated with exogenous C2-ceramide or C2-dihydroceramide, LY-R cells were seeded at 1.5–2 × 10^5 cells/ml in T-25 flasks, and a lipid or DMSO was added to each flask for given periods of time. DMSO (1% final concentration) had no effect on either cell viability or responsiveness of cells.

Before the exposure of cells to hydrogen peroxide (1 mM), they were either serum-starved (U937 for 20 h) or grown overnight in LSM (LY-R). In the latter case, LY-R cells were transferred to Fisher's medium, preincubated for 1 h at 37°C, and then treated with hydrogen peroxide for desired periods of time.

Measurement of Ceramide Level. Ceramide mass was determined using a protocol described previously (10, 16, 17). Cell suspensions were transferred to prechilled glass tubes and centrifuged (2000 rpm, 10 min, 4°C), and the pellets [(7.4 ± 0.7) × 10^6 cells, mean ± SE; n = 18] were resuspended in cold 1 M NaCl (1 ml) and chloroform/methanol (1:2, v/v; 3.75 ml) and mixed. Lipid phases were separated by further addition of chloroform (1.25 ml) and NaCl (1 M, 1.25 ml). An aliquot (2.2 ml) of the lower phase was transferred to a 15-ml glass tube and dried under nitrogen. The lipid film was dissolved in 0.1 M KOH/methanol (1 ml), and the tubes were capped and incubated in a shaking water bath at 37°C for 1 h. After alkaline hydrolysis, the remaining phospholipids were extracted with chloroform (1 ml) and water (0.6 ml). An aliquot (0.9 ml) of the lower phase was transferred to a 12 × 75-mm glass tube, and a lipid mixture (1 ml/sample) containing octylglucoside and cardiolipin in methanol (at final concentrations of 52 mM and 1.5 mg/ml, respectively) was added. After vortexing, samples were dried under nitrogen. After the addition of 0.13 ml of a reaction mixture [50 mM imidazole (pH 6.6), 12.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaCl, and 80 μM DETAPAC] and 3 μl of freshly

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[3] The abbreviations used are: C2-ceramide, *N*-acetyl-sphingosine; C2-dihydroceramide, *N*-acetyl-dihydrosphingosine; DAG, diacylglycerol; DETAPAC, diethylenetriaminepentaacetic acid; LED, light-emitting diode; LSM, low-serum medium; LY-R, L5178Y-R; PDT, photodynamic treatment; SAPK, stress-activated protein kinase; JNK, c-Jun kinase; TLC, thin layer chromatography.

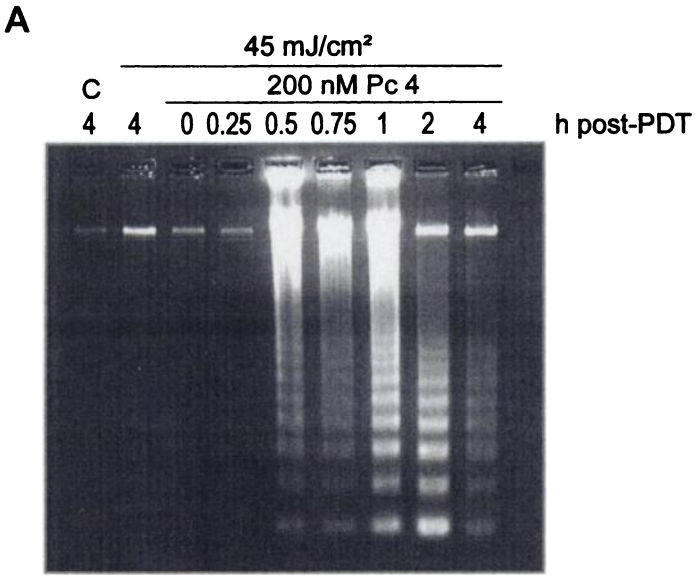
prepared 100 mM DTT to each sample, the tubes were vortexed, and the reaction was started by the addition of a solution containing 1 mM ATP, 0.9 μCi [γ-^{32}P]ATP (4500 Ci/mmol), and 1 μg of DAG kinase (3.13 units/mg). The samples were incubated at room temperature for 1 h, and then the lipids were extracted with chloroform/methanol (1:2, 0.6 ml), 0.2 ml of chloroform, and 0.2 ml of 1% HCl. An aliquot (0.3 ml) of the lower phase was transferred to a fresh tube, dried under nitrogen, resuspended in chloroform/methanol (1:1, v/v; 0.05 ml), and spotted onto a TLC plate. Plates were developed for 75 min in chloroform/methanol/acetone/glacial acetic acid (50:15:20:10, v/v), air-dried, and left overnight in a phosphorimager cassette. A PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA) was used for analysis of samples. To quantify the amount of radioactivity in samples, known amounts of [^{32}P]ATP were spotted on each plate. The spots containing ceramide phosphate were identified using ceramide standard. Loss of ceramide, as assessed by the recovery of ceramide standard, was 15% throughout various procedural steps. To confirm that cell numbers analyzed in each sample of each TLC plate were identical, the intra-assay variability in cell number, as expressed by the covariance, was calculated for two separate experiments and was found to be 10.9% (n = 13) and 9.1% (n = 12), respectively.

DNA Purification and Gel Electrophoresis. After treatments and incubations, total cellular DNA was isolated and analyzed on a 1.5% agarose gel, as described elsewhere (9).

Flow Cytometry. The procedure for cell fixation and staining has been described (18, 19). Flow cytometric analysis was carried out in the Flow Cytometry Facility of the Case Western Reserve University/Ireland Cancer Center.

RESULTS AND DISCUSSION

The induction of apoptosis by PDT is dependent on light fluence and photosensitizer concentration (8). For the present study, the PDT dose was varied by increasing the concentration of Pc 4 (50–300 nM), and after a 13–19-h incubation to allow uptake of the photosensitizer, LY-R cells were irradiated with a constant fluence of red light delivered by an LED light source (1.5 mW/cm^2, 45 mJ/cm^2, 30 s). DNA fragmentation, cell viability, and clonogenicity were assessed after these treatments. Although oligonucleosomal DNA fragmentation was observed 2 h after each of the three PDT doses (data not shown), it was most pronounced after PDT with 200 nM Pc 4. In addition, all cells became nonviable (*i.e.*, trypan blue-positive) by 2 h after PDT with 300 nM Pc 4, suggesting that this was a highly supralethal PDT dose. PDT with 200 nM Pc 4 was selected for further study. This dose was found to kill 99.9% of the cells (surviving fraction, 0.001), as judged by a clonogenic assay, and, as shown in Fig. 1*A*, to induce DNA fragmentation, a qualitative measure of apoptosis, by 30 min post-PDT.



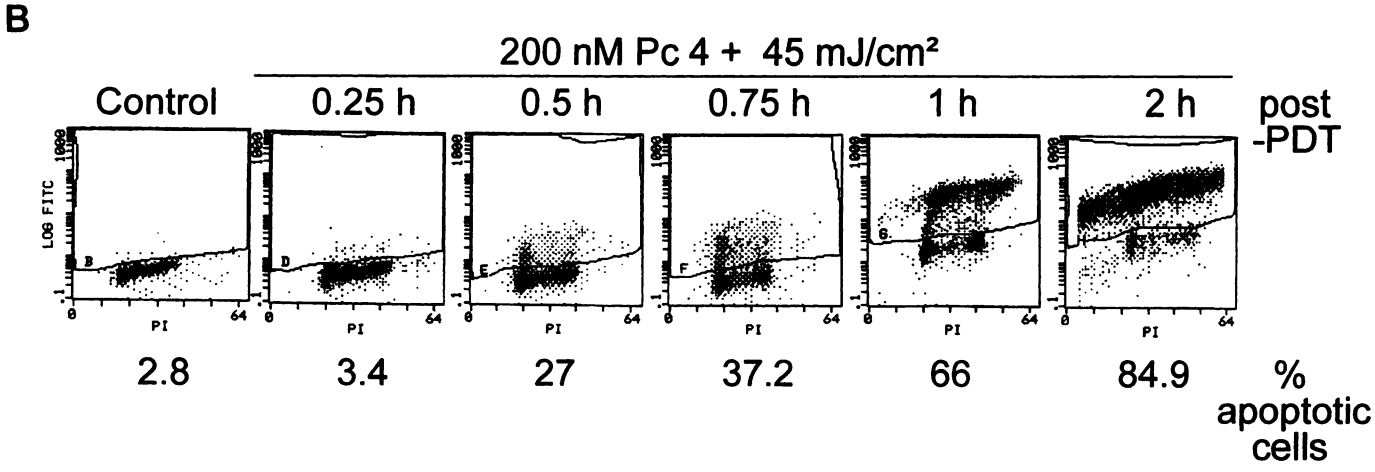


Fig. 1. Time course of PDT-induced apoptosis in LY-R cells, as estimated by DNA fragmentation and flow cytometry. The cells were treated with 200 nM Pc 4 and 45 mJ/cm^2 red light. At the indicated times postirradiation, cells were collected and analyzed for apoptosis. *A*, total DNA was isolated and size-separated on a 1.5% agarose gel; *lane C*, untreated control. Similar data were obtained in two additional experiments. *B*, cells were labeled with FITC-dUTP using terminal deoxynucleotide transferase and with propidium iodide and analyzed by flow cytometry. Cells displaying FITC fluorescence above that of the control population, as indicated by the *line* in each histogram, are considered apoptotic. Similar data were obtained in another independent experiment.

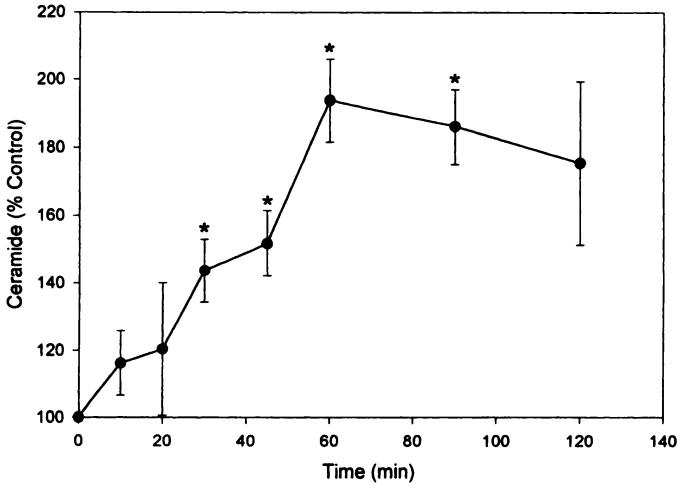


Fig. 2. Time course of ceramide generation in response to PDT in LY-R cells. After PDT treatment (200 nM Pc 4, 45 mJ/cm^2), the cultures were incubated for the indicated times. Lipids were extracted, and ceramide was determined using a modified DAG kinase assay. The control ceramide levels were 9 ± 1 pmol/10^6 cells (mean ± SE; n = 17). Data were calculated as the percentage of time-matched, light-treated controls analyzed in the same experiment and are shown as the mean ± SE of four to eight separate determinations (except for the 120-min time point, which is the average of two determinations). *, significantly different from corresponding control ($P < 0.01$, paired Student t test).

To quantify the time course and extent of apoptosis in PDT-treated LY-R cells, DNA termini were tagged with FITC-labeled dUTP in a reaction catalyzed by terminal deoxynucleotidyl transferase, DNA was stained with propidium iodide, and the cells were analyzed by flow cytometry. As shown in Fig. 1*B*, for PDT with 200 nM Pc 4, 27% of the cells were undergoing apoptosis by 30 min after PDT, and the apoptotic population reached nearly 85% in 2 h. For the 1-h and 2-h populations, nearly all cells had FITC fluorescence above the control level, but only the population with the highest level was considered apoptotic. In contrast, for PDT with 100 nM Pc 4, 2 h were required for 25% of the cells to become apoptotic (data not shown). Thus, both the flow cytometry and the DNA fragmentation assay demonstrated that the apoptotic response of LY-R cells to PDT was dose- and time-dependent.

The time course for ceramide generation was assessed after exposure of LY-R cells to PDT with 200 nM Pc 4. Although there was a trend toward an increase in ceramide as early as 10 and 20 min post-PDT, the elevation of this lipid did not reach the level of significance until 30 min (Fig. 2). Ceramide was increased above the basal level by 44, 52, 94, 86, and 75% at 30, 45, 60, 90, and 120 min, respectively. Therefore, it seems that PDT led to a time-dependent accumulation of ceramide in LY-R cells. The observed increases in the steady-state level of ceramide are significant, considering that it is the central molecule in the biosynthesis and breakdown of sphingolipids (20). Because the time courses for ceramide synthesis and for apoptosis overlap, it is possible that ceramide generation is required for PDT-induced apoptosis.

Similar elevations in ceramide levels have been observed after other oxidative stresses (12). Correspondingly, we found a 109 and 80% increase in ceramide production in response to H_2O_2 (1 mM) in U937 cells at 1 and 5 min, respectively (data not shown). Moreover, ceramide levels were elevated in response to the same stress inducer in LY-R cells by 119 and 107% above resting levels at 1 and 5 min, respectively (data not shown). Thus, it seems that ceramide synthesis is stimulated in LY-R cells in response to more than one stress.

To assess the PDT dose dependence for ceramide accumulation, LY-R cells were exposed to different doses of Pc 4 overnight, irradiated, and then incubated for 60 min. As shown in Fig. 3, ceramide levels increased with PDT dose, reaching near maximum levels after PDT with 100 nM Pc 4. Moreover, the ED_{50} (46 nM) was virtually the same as the LD_{50} (40 nM). These results support but do not prove a link between increased ceramide generation and cell death in the response of LY-R cells to PDT.

To test whether exogenous ceramide can mimic the apoptotic effect of PDT in LY-R cells, both dose- and time-dependency studies were undertaken with C2-ceramide, a cell-permeable, biologically active form of ceramide (20). At low concentrations (0.1 or 1 μM) of C2-ceramide, there was no observable DNA fragmentation at 6, 16, or 24 h (data not shown), and the cells remained viable, as assessed by trypan blue exclusion. When LY-R cells were exposed to high concentrations (30 or 100 μM) of C2-ceramide, they became nonviable within 2 h of treatment and did not undergo apoptosis, as indicated by the absence of DNA fragmentation (data not shown). These concentrations were not studied further because of their high toxicity. At an intermediate concentration (10 μM) of C2-ceramide, oligonucleosomal DNA fragmentation was detectable at 12, 16, and 24 h, but not at earlier times (Fig. 4). The same concentration (10 μM) of C2-dihydroceramide, a biologically inactive ceramide analogue (21), did not initiate DNA fragmentation within 16 h, suggesting that the induction of apoptosis was specific for the active C2-ceramide. Thus, exogenous C2-ceramide was able to trigger apoptotic changes in LY-R cells at a 10-fold lower concentration than the one used by Verheij *et al.* (12) in U937 and bovine aortic epithelial cells. It seems that the dose response for the induction of apoptosis by C2-ceramide is cell line-dependent.

Curiously, the C2-ceramide-induced cell killing occurred on a longer time scale than that of PDT-induced apoptosis (earliest observable DNA fragmentation at 12 h *versus* 30 min, respectively). However, structural differences in ceramides are known to be associated with differences in biological effectiveness. For example, one of the actions of C8-ceramide, phosphorylation of the epidermal growth factor receptor on threonine residues, cannot be mimicked by C2-ceramide (22). Therefore, we suggest that the exogenous short-chain C2-ceramide may have used a different route for induction of apoptosis than the endogenously produced long-chain ceramide. For instance, C2-ceramide may require new RNA/protein synthesis for the initiation of apoptosis in LY-R cells (23), whereas PDT does not (2). In addition, a possibility that ceramide generation is a consequence of

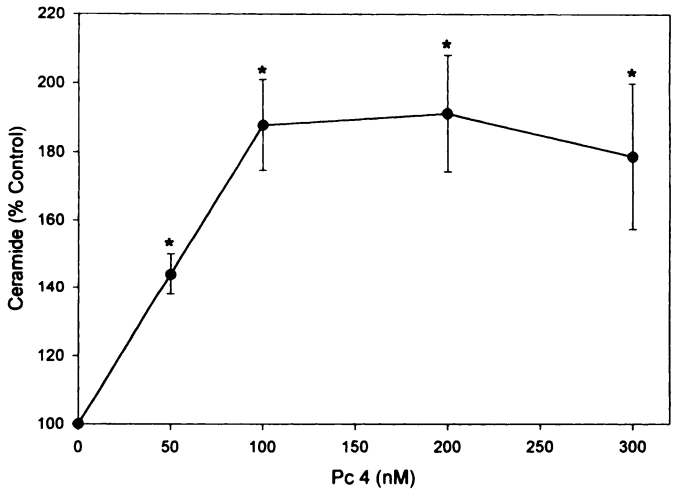


Fig. 3. PDT dose response for ceramide generation in LY-R cells. After the treatment of cells with the indicated concentrations of Pc 4, the cultures were irradiated (45 mJ/cm^2) and then incubated for 60 min. Ceramide levels were calculated as the percentage of time-matched, light-treated controls run in the same experiment and are shown as the mean ± SE for five to seven independent determinations. *, significantly different from time-matched, light-treated controls ($P < 0.02$, paired Student t test).

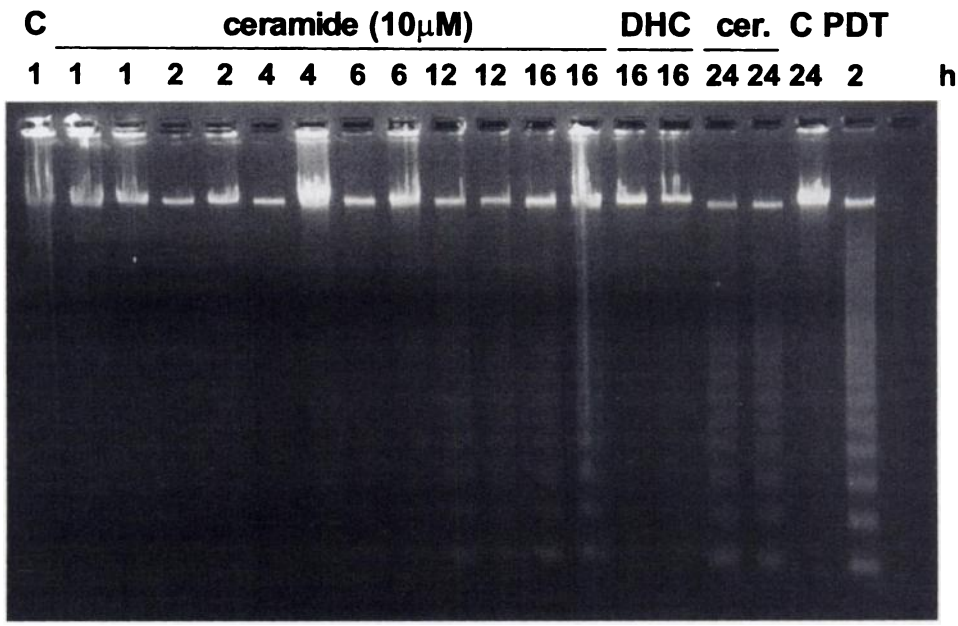


Fig. 4. Kinetics of C2-ceramide-induced DNA fragmentation in LY-R cells. Exogenous C2-ceramide or C2-dihydroceramide was added to the cultures to a final concentration of 10 μM. After the indicated times, DNA was isolated and analyzed by agarose gel electrophoresis. Similar results were obtained in two additional experiments. *C*, control; *DHC*, C2-dihydroceramide; *Cer*, C2-ceramide. The PDT sample was obtained 2 h after PDT with 200 nM Pc 4.

apoptosis, rather than a trigger for apoptosis, cannot be excluded. This proposal is in agreement with the notion that ceramide plays a role as a biostat that detects and regulates responses to cellular stresses (22).

Cellular stresses elicit ceramide generation, leading to activation of the SAPK/JNK signaling pathway (11, 12, 24), which in turn is responsible for activation of transcription factors, such as c-jun (12, 25), and stimulation of gene expression. However, it is unlikely that the later steps of this pathway participate in signaling PDT-induced apoptosis in LY-R cells because: (*a*) the apoptotic response to PDT is a rapid event (initiating within 30 min); (*b*) new gene expression is not required (2); and (*c*) although the SAPK/JNK cascade is activated in PDT-treated mouse keratinocytes (13), it does not seem to respond similarly to PDT in LY-R cells.[4] If ceramide is at least partially responsible for mediating apoptosis in PDT-treated LY-R cells, it is clear that downstream targets other than transcription factors must be involved. Possible candidates for ceramide targets could be ceramide-activated protein phosphatase (26) and a proline-directed protein kinase (27).

In this study, we reported that PDT of LY-R cells leads to elevated ceramide levels. Whether the ceramide accumulation is a result of sphingomyelinase activation (10, 28) or increased *de novo* ceramide synthesis (29) has not yet been established. An involvement of sphingomyelinase in the stimulated production of ceramide implies that the plasma membrane is a target for PDT because the sphingolipid content of the plasma membrane is generally greater than that of other cellular membranes (30). This notion is supported by the finding that phospholipases A_2 and C are activated in response to PDT in LY-R cells (9). The similarity in timing between the increase in ceramide and the onset of apoptosis in response to PDT and the close correlation between the ED_{50} and the LD_{50} support the hypothesis that ceramide generation is associated with PDT-induced early apoptotic cell death. In contrast, the delayed apoptosis in response to exogenous C2-ceramide may be a consequence of the activation of an alternative route for the induction of apoptosis.

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[4] L-y. Xue and N. L. Oleinick, unpublished observations.

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