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Ceramide response post-photodamage is absent after treatment with HA14-1

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Abstract

The oxidative stress induced by photodynamic therapy using the phthalocyanine Pc 4 (PDT) can lead to apoptosis, and is accompanied by photodamage to Bcl-2 and accumulation of de novo ceramide. Similar to PDT, the oxidative stress inducer and Bcl-2 inhibitor HA14-1 triggers apoptosis. To test the specificity of the ceramide response, Jurkat cells were exposed to an equitoxic dose of HA14-1. Unlike PDT, HA14-1 did not induce accumulation of de novo ceramide, although levels of sphingomyelin, phosphatidylserine and phosphatidylethanolamine were below control values after either treatment. In contrast to PDT, (i) the transient inhibition of serine palmitoyltransferase induced by HA14-1 was associated with the initial decrease in de novo ceramide, and (ii) HA14-1-initiated inhibition of sphingomyelin synthase and glucosylceramide synthase did not result in accumulation of de novo ceramide. These results show that the ceramide response to PDT is not induced by another pro-apoptotic stimulus, and may be unique to PDT as described here.

Keywords

Apoptosis; Ceramide; Glucosylceramide synthase; HA14-1; PDT; Serine palmitoyl-transferase; Sphingomyelin synthase

Generation of ceramide in the de novo biosynthetic pathway can be induced by various agents, including anti-cancer drugs and radiation [1–4]. Induced de novo ceramide production during apoptosis includes activation of serine palmitoyltransferase (SPT) [5], the enzyme that catalyzes the first reaction in the ceramide biosynthetic pathway, as well as inhibition of glucosylceramide synthase (GCS), the enzyme that converts de novo ceramide to glucosylceramide [4,6]. Activation of sphingomyelin synthase (SMS), the enzyme catalyzing conversion of ceramide to sphingomyelin, in addition to activation of GCS, has been implicated in decrease in ceramide and chemoresistance [7]. In addition, a correlation between the antiapoptotic protein Bcl-2 and a decrease in ceramide levels has been reported [2,8–10]. Inostamycin-induced de novo ceramide accumulation during apoptosis involves Bcl-2 [2]. However, it is unclear what effect Bcl-2 has on SPT, SMS and GCS activities.

The oxidative stress after photodamage with the silicon phthalocyanine Pc 4 (PDT) can result in apoptosis [11,12], and is associated with Bcl-2 photodamage [13,14] and de novo ceramide accumulation [15]. The ceramide response occurs in the absence of SPT upregulation, and is a result of inhibition of ceramide-metabolizing enzymes SMS and GCS [15]. To test whether the mechanism of de novo ceramide accumulation is unique to PDT, we compared PDT to HA14-1, a cell permeable non-peptidic inhibitor of Bcl-2 function [16] and an oxidative stress inducer [17–19].

Materials and methods

Materials

The phthalocyanine photosensitizer Pc 4, $\text{HOSiPcOSi}-(\text{CH}_3)_2(\text{CH}_2)_3\text{N}(\text{CH}_3)_2$, was supplied by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). HA14-1 was obtained from Ryan Scientific and Sigma–Aldrich. Ac-DEVD-AMC was purchased from Biomol. Hoechst 33342 dye was purchased from Molecular Probes. Basic media and sera were from Invitrogen and Hyclone, respectively. Cell proliferation kit (MTT) was from Roche Applied Science. Thin layer chromatography (TLC) plates (aluminum sheets of silica gel 60) were from EM Science. Other chemicals were from Sigma–Aldrich and J.T. Baker.

Cell culture and treatments

Jurkat, clone E6-1 cells were purchased from American Type Culture Collection. Jurkat cells were cultured in RPMI 1640 medium (supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin) and were maintained at 37 °C in a 5% CO_2 atmosphere. For both HA14-1 and PDT experiments, cells were treated in growth medium and all incubations were performed at 37 °C in a 5% CO_2 atmosphere. For PDT experiments, after overnight incubation with Pc 4 (200 nM), cells were irradiated with red light (2 mW/cm^2 ; $\lambda_{\text{max}} \sim 670 \text{ nm}$) using a light-emitting diode array light source (EFOS) at various fluences at room temperature.

DEVDase activity

The enzyme activity was measured as described previously [15]. Caspase activity was determined by its ability to cleave the fluorogenic derivative (Biomol) 7-amino-4-methylcoumarin (AMC) of the tetrapeptide substrate *N*-acetyl-Asp-Glu-Val-Asp (DEVD). An aliquot of cytosol (50 μg) was incubated with the caspase substrate DEVD-AMC in buffer supplemented with EDTA, EGTA, dithiothreitol, and protease inhibitors for 1 h at 37 °C. The released fluorescence of the cleaved DEVD substrate was measured in a F-2500 Hitachi spectrofluorometer (380 nm excitation and 460 nm emission).

Hoechst staining

Nuclear chromatin condensation was determined by staining with the DNA-binding Hoechst 33342 dye as described previously [15]. An aliquot of formalin-fixed cells was examined by fluorescence microscopy (Zeiss).

Cell viability

Cytotoxicity was assessed using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; MTT) assay according to manufacturer's instructions. The assay is based on the ability of metabolically active cells to cleave yellow tetrazolium salt MTT to purple formazan crystals. Following treatments, cells ($4 \times 10^5/\text{ml}$) were placed into individual wells of a 96-well plate, incubated for 48 h, exposed to MTT, and incubated for additional 4 h. The reaction was stopped following overnight exposure to sodium dodecyl sulfate-containing solubilization solution.

The absorbances of solubilized formazan crystals were determined at 550 nm using SpectraMax M2 Microplate Reader (Molecular Devices). The reference wavelength was 690 nm.

Metabolic radiolabeling of cellular lipids

Pulse labeling was performed as reported previously [15]. Following PDT, cells were labeled with [^{14}C]serine (28 kBq; GE Healthcare) in the culture medium for 2 h. Afterwards, cells were harvested and collected by centrifugation at 4 °C. Total extracted cellular lipids were separated by TLC (methyl acetate/*n*-propanol/chloroform/methanol/0.25% potassium chloride; 25/25/25/10/9; v/v). After chromatography, the TLC plates were exposed to phosphor-imager screens (GE Healthcare) for 48 h. The samples were analyzed by a STORM 860 imaging system (GE Healthcare).

Preparation of microsomal membranes

Microsomal membranes were prepared as described previously [15,20]. All steps were performed on ice. Cells (25×10^6) were centrifuged, washed with PBS, and resuspended in 500 μl of homogenization buffer (20 mM Hepes, pH 7.4, 5 mM dithiothreitol, 5 mM EDTA, 2 $\mu\text{g/ml}$ leupeptin, and 20 $\mu\text{g/ml}$ aprotinin). Cells were disrupted at 20% output, alternating a 15 s sonication with a 20 s pause for four cycles using a VirSonic 100 dismembrator (Virtis). Lysates were centrifuged at 10,000g for 10 min. The postnuclear supernatant was centrifuged at 250,000g for 30 min. The microsomal pellet was resuspended in 250 μl of freezing buffer (10 mM Hepes, pH 7.4, 250 mM sucrose) using 26 gauge needle. Membranes were frozen (-80°C) until use.

SPT activity assay

As described previously [21,15], the SPT activity in 100 μg of microsomal membranes was measured in 50 mM Hepes (pH 7.5), 5 mM dithiothreitol, 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 l -[^3H]serine (GE Healthcare; 1 mM 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 0.5 N NH_4OH . The [^3H]-labeled lipid product 3-ketosphinganine was extracted and the radioactivity was measured by scintillation counting. SPT activity was expressed as picomoles 3-ketosphinganine generated per 10 min per 1 mg protein after subtracting the background radioactivity (i.e., the minus palmitoyl-CoA control).

Sphingomyelin and glucosylceramide synthase activity assay

The SMS activity assay using *N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine (C6-NBD-ceramide) was performed as described previously [15,22]. Enzyme activity in 50 μg of microsomal membranes was measured in 50 mM Hepes (pH 7.5), 5 mM EDTA, and 10 μM C6-NBD-ceramide complexed with fatty acid-free bovine serum albumin (0.1 mM). To determine the GCS activity, 500 μM UDP-glucose was included in the assay mixture [22]. Following incubation for 5 min at 37 °C, the reaction was terminated with chloroform/methanol (1/2, v/v). C6-NBD-ceramide-labeled lipid products were extracted, 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. SMS and GCS activities were expressed as picomoles sphingomyelin and glucosylceramide, respectively, generated per 5 min per 1 mg protein after subtracting the background fluorescence.

Statistical analysis

Results were expressed as means $\pm$ SE. Statistical analyses were performed by Student's *t* test. Significance was defined as a two-tailed *p* < 0.05.

Results and discussion

Cytotoxicity

To compare the effects of the two treatments, equitoxic doses of HA14-1 and PDT were used. To assess cytotoxicity of the two treatments, MTT assay was employed in dose-response studies. For PDT, Pc 4 concentration was kept at 200 nM, while light fluences were varied. When Pc 4-treated Jurkat cells were exposed to 68, 135, and 200 mJ/cm² fluences, 9, 37, and 67% of the cells, respectively, were killed. As the fluences were increased to 270 and 400 mJ/cm² in the presence of Pc 4, a 93 and 99.9% reduction in cell survival, respectively, was observed (Fig. 1A). In contrast, incubation of Jurkat cells with HA14-1 (0.3 or 3 μM) had virtually no effect on cell survival. At 10 μM HA14-1, a 24% decrease in cell survival was detected. However, in the presence of 20 and 30 μM HA14-1, a substantial drop in the survival was observed, since 94 and 99% of cells, respectively, were killed (Fig. 1B). For the subsequent comparative studies, ~LD₉₉ dose of either treatment was used.

Apoptosis

We have already shown that Jurkat cells undergo apoptosis post-Pc 4-PDT [12,23], and that HA14-1 induces apoptosis in murine leukemia L1210 cells [18,24]. In this study, HA14-1-induced apoptosis was assessed in Jurkat cells using DEVDase assay and Hoechst nuclear staining. Incubation with HA14-1 (30 μM) for 0.5, 1, or 2 h, led to 2.1 ± 0.4 , 4.8 ± 0.9 , and 19.1 ± 2.8 -fold increases over control in DEVDase activity, respectively (Fig. 1C). Under the same conditions apoptosis was observed in 4 ± 1 , 10 ± 4 , and $48 \pm 3\%$ of cells, respectively (Fig. 1D). The data demonstrate that in Jurkat cells HA14-1 also induces apoptosis.

Ceramide accumulation is not induced post-HA14-1

We have shown that PDT triggers de novo ceramide accumulation in Jurkat cells [15]. Here we tested whether exposure of Jurkat cells to HA14-1 leads to the ceramide response. Treatment of cells with HA14-1 (30 μM) for 0.5 h resulted in reduction of basal [¹⁴C]ceramide levels by 30% (Fig. 2A). At 1 or 2 h post-HA14-1 (30 μM) there was no significant change in control [¹⁴C]ceramide levels (Fig. 2A). Similarly, at lower HA14-1 doses (10 and 20 μM) there was no change in the lipid at 2 h (not shown). In contrast, exposure of cells to PDT (200 nM Pc 4 + 400 mJ/cm²) led to a 2.84 ± 0.82 -fold increase in [¹⁴C]ceramide at 2 h (Fig. 2A).

Exposure of Jurkat cells to HA14-1 (30 μM) for 0.5, 1 or 2 h, led to a substantial decrease in resting levels of [¹⁴C]sphingomyelin, [¹⁴C]phosphatidylserine and [¹⁴C]phosphatidylethanolamine (Fig. 2 B, C, D). We observed similar trends in these lipids after HA14-1 at lower doses (10 and 20 μM) at 2 h (not shown). The responses of [¹⁴C]sphingomyelin, [¹⁴C]phosphatidylethanolamine, and [¹⁴C]phosphatidylserine to PDT were similar to those of HA14-1 (Fig. 2 B, C, D). The data show that the absence of de novo ceramide accumulation post-HA14-1 was the only difference in lipid responses between the two treatments.

Enzyme activities

We have demonstrated that SPT is not upregulated in Jurkat cells after PDT [15]. In the next series of experiments we measured SPT activity in microsomes isolated from either control- or HA14-1-treated Jurkat cells. Treatment of cells with HA14-1 (30 μM) for 0.5 h resulted in attenuation of the enzyme activity by 33% (Fig. 3A). This effect was paralleled by transient decrease in de novo ceramide below control levels (Fig. 2A). At 1 or 2 h post-HA14-1 (30 μM), however, there was no significant change in SPT activity (Fig. 3A). Accordingly, de novo ceramide remained at resting levels (Fig. 2A). At lower HA14-1 doses, 10 and 20 μM, the

enzyme was inhibited and activated, respectively (not shown). Under those conditions there was no correlation between SPT activity and ceramide (not shown).

We have shown that PDT induces profound inhibition of SMS and GCS [15]. The activities of SMS and GCS were determined in microsomes isolated from either control- or HA14-1-treated Jurkat cells. Exposure of cells to HA14-1 (30 μ M) for 2 h resulted in reduction of the enzyme activity by 58% (Fig. 3B). This effect correlated with reduction in [14 C]sphingomyelin below control levels (Fig. 2B). However, there was no correlation between [14 C]sphingomyelin and SMS activity under other conditions. Moreover, GCS was inhibited at all time points post-HA14-1 (30 μ M; Fig. 3C) or other HA14-1 doses (10 μ M and 20 μ M) that we tested (not shown). Thus, contrary to PDT, HA14-1-induced inhibition of SMS and GCS did not result in accumulation of de novo ceramide.

The present study shows that: (i) compared to HA14-1, de novo ceramide accumulation is a distinct response to PDT in Jurkat cells; (ii) in contrast to PDT, HA14-1-induced inhibition of SPT is involved in the initial decrease in de novo ceramide; (iii) contrary to PDT, HA14-1-induced inhibition of SMS and GCS did not result in accumulation of ceramide. The data support a role of other enzymes in determining the overall de novo ceramide levels post-HA14-1.

In the previous study we observed [18] that treatment of L1210 murine leukemia cells with HA14-1 or PDT, targeting either the ER or lysosomes, led to MAP kinase activation and reactive oxygen species (ROS) production, indicating that these phenomena were not unique to PDT. The present study shows for the first time that de novo ceramide accumulation is not induced by HA14-1 in Jurkat cells. In comparison to PDT with Pc 4, targeting primarily mitochondria, but also the ER and Golgi complex [11], this was the only distinct lipid response to HA14-1. We have shown both pharmacologic [25,26] and genetic [27] evidence that PDT-induced ceramide production is independent of caspases, and that PDT-induced ROS production is independent of de novo sphingolipid production [12]. Overall, our data suggest that the ceramide response to PDT is induced by oxidative stress rather than by apoptosis.

Reportedly, upregulation of Bcl-2 is associated with a decrease in de novo ceramide with subsequent inhibition of apoptosis post-inostamycin [2], supporting the role of ceramide in apoptosis. Similarly, we have provided both genetic and pharmacologic evidence supporting the notion that de novo ceramide is associated with promotion of apoptosis after PDT [23, 28]. However, the present study shows that HA14-1-induced inhibition of Bcl-2 leading to apoptosis is not sufficient to induce the ceramide response, suggesting that additional PDT effects are involved in the response.

The data from the present comparative study provide new evidence supporting the view that the mechanism of de novo ceramide accumulation is a unique lipid response during PDT-induced apoptosis in Jurkat cells. Because co-exposure to PDT and C16-ceramide enhances apoptosis [12], understanding of mechanisms underlying the ceramide response to photodamage may lead to the development of methods designed to augment PDT efficiency.

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Fig. 1.

Cytotoxicity of PDT and HA14-1 [29]. (A,B) Jurkat cells were irradiated at the indicated fluences after overnight incubation with Pc 4 (200 nM) (A), or exposed to the indicated doses of HA14-1 for 2 h (B). Cell viability was determined by MTT assay. Controls consisted of light, Pc 4, DMSO, or no treatment. Light itself, Pc 4 or DMSO alone had no effect on cell viability. The data are shown as means $\pm$ SE for two-three independent determinations. (C,D) Following treatment of Jurkat cells with HA14-1 (30 μ M) for the indicated periods of time, DEVDase activity was measured spectrofluorimetrically in cell lysates using DEVD-AMC as the substrate (A) or nuclear apoptotic changes were detected by Hoechst staining using fluorescence microscopy (B). The data are shown as means $\pm$ SE for six-eight independent determinations. Con, DMSO-treated cells. *Different ($P < 0.05$) from DMSO-treated controls.

Fig. 2.

The effects of HA14-1 and PDT on [^{14}C]lipids. Jurkat cells were exposed to [^{14}C]serine immediately after HA14-1 (30 μM) or PDT (200 nM Pc 4 + 400 mJ/cm^2) for the indicated periods of time. Following incubations, cells were harvested, lipids were extracted, and [^{14}C]-labeled lipids were analyzed by TLC and phosphorimaging. (A) [^{14}C]Ceramide; (B) [^{14}C]sphingomyelin; (C) [^{14}C]phosphatidylserine; (D) [^{14}C]phosphatidylethanolamine. The data are expressed as -fold change relative to DMSO and Pc 4 control for HA 14-1- and PDT-treated cells, respectively. The data are shown as the mean $\pm$ SE for six-ten independent determinations. The resting levels of [^{14}C]ceramide, [^{14}C]sphingomyelin, [^{14}C]phosphatidylserine, and [^{14}C]phosphatidylethanolamine were 3353 ± 495 , 4740 ± 521 , 92708 ± 13732 , 12001 ± 1659 pixels. *Different ($P < 0.05$) from DMSO- and Pc 4-treated controls for HA14-1- and PDT-treated cells, respectively.

Fig. 3.

The effects of HA14-1 and PDT on serine palmitoyltransferase, sphingomyelin synthase and glucosylceramide synthase activities. In PDT experiments, Jurkat cells were treated overnight with Pc 4 (200 nM), irradiated at the indicated light fluences and then incubated for 1 h [15]. In HA14-1 experiments, cells were exposed to the drug (30 μ M) or DMSO for the indicated periods of time. After harvesting the cells, microsomes were isolated and the activities of the enzymes were assayed as described in the Methods. (A) Serine palmitoyltransferase; (B) sphingomyelin synthase; (C) glucosylceramide synthase. The data are expressed as -fold change relative to untreated controls and are shown as means $\pm$ SE of four to eight independent determinations. The resting levels of serine palmitoyltransferase were 61 ± 6 pmol/mg protein/10 min. For sphingomyelin synthase and glucosylceramide synthase, the basal activity levels were 186 ± 46 and 1209 ± 879 pmol/mg protein/5 min, respectively. *Different ($P < 0.05$) from DMSO- and Pc 4-treated controls for HA14-1- and PDT-treated cells, respectively.