Biochemical and Biophysical Research Communications 458 (2015) 456–461

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential apoptotic effect and metabolism of N-acetylsphingosine and N-hexanoylsphingosine in CHP-100 human neurotumor cells

Sabrina Di Bartolomeo, Antonio Agostini, Angelo Spinedi*

Department of Biology, University of Rome 'Tor Vergata', Via della Ricerca Scientifica, 00133 Rome, Italy

ARTICLE INFO

Article history:
Received 14 January 2015
Available online 3 February 2015

Keywords:
N-acetylsphingosine
N-hexanoylsphingosine
Long-chain ceramide
Sphingosine salvage pathway
Apoptosis

ABSTRACT

The cytotoxic effects of N-acetylsphingosine (C2-Cer) and N-hexanoylsphingosine (C6-Cer) were compared together with their specific intracellular accumulation profiles and metabolism in human CHP-100 neuroepithelioma cells. The two short-chain ceramides, administered in the culture medium at an equimolar concentration, evoked a differential apoptotic response, with C6-Cer showing markedly more cytotoxic than C2-Cer. Apoptosis, that was suppressed in both cases by inhibition of caspase-9, but not of caspase-8, associated with a higher intracellular accumulation of C6-Cer over C2-Cer, notwithstanding C6-Cer was actively metabolized by direct glucosylation or by conversion to natural ceramide via the sphingosine salvage pathway, whereas C2-Cer was apparently metabolically inhert. C2-Cer cytotoxicity was markedly enhanced by increasing its concentration in the culture medium, and this response associated with a higher intracellular accumulation of this compound, in the absence of any natural ceramide elevation. These results support the notion that the differential apoptotic effect evoked by C2-Cer and C6-Cer in CHP-100 cells is driven by their differential intracellular accumulation profiles, but not by their differential property to generate natural ceramide via the sphingosine salvage pathway.

1. Introduction

Short-chain ceramides are emerging as potential pharmacological agents, due their anti-proliferative and pro-apoptotic properties: N-hexanoylsphingosine (C6-Cer), in particular, is currently under evaluation as an anticancer agent, after preclinical studies showing its property to reduce tumor growth in vivo [1–4]. It is well established that potentiation of the apoptotic response evoked by C6-Cer can be achieved by limiting its metabolic processing to non-toxic derivatives, in particular glucosylceramide (GlcCer) [5,6]; moreover, cells may utilize C6-Cer for natural (i.e. Long-Chain) ceramide (LC-Cer) synthesis via the sphingosine salvage pathway, namely via deacylation and reacylation of the sphingosine backbone with a long-chain fatty acid [7–9]. The functional significance of the latter metabolic route largely remains however to be explored and, as far as apoptosis induction is concerned, studies so far performed indicate that it may be cell-type-specific [10,11].

N-Acetylsphingosine (C2-Cer) has also been widely utilized to induce apoptosis in *in vitro* studies; however, observations performed on various cell systems demonstrated that the length of the acyl chain linked to the sphingosine backbone may confer different apoptotic properties to short-chain ceramides, with C6-Cer showing generally more cytotoxicity than C2-Cer [10,12,13]. On the one hand, it has been envisaged that the above-mentioned phenomenon mainly impinges on the optimal amphiphilic properties of C6-Cer, leading to a higher intracellular accumulation over C2-Cer, that is somewhat more hydrophilic [12,13]; however, evidence has also been provided that the differential apoptotic effect of the two short-chain ceramides may depend on the extent of their conversion to LC-Cer via the sphingosine salvage pathway, with C2-Cer being generally utilized by cells less efficiently than C6-Cer, along this metabolic route [10].

Previous studies from this laboratory showed that C6-Cer induces apoptosis in CHP-100 human neurotumor cells, as well a generation of LC-Cer via the salvage pathway [5]; however, it was also demonstrated that the ceramide synthase inhibitor fumonisin B1 was ineffective in rescuing cells from C6-Cer-induced cell death, thus suggesting that LC-Cer produced via the above-mentioned

Abbreviations: C2-Cer, N-acetylsphingosine; C6-Cer, N-hexanoylsphingosine; LC-Cer, long-chain ceramide; GSLs, glycosphingolipids; GlcCer, glucosylceramide; HPTLC, high-performance thin layer chromatography; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; Z-LEHD.fmk, N-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone; Z-IETD.fmk, Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) fluoromethylketone.

* Corresponding author. Fax: +39 062023500.
E-mail address: spinedi@uniroma2.it (A. Spinedi).

http://dx.doi.org/10.1016/j.bbrc.2015.01.107
0006-291X/

metabolic route is dispensable for apoptosis induction [11]. On this ground, the present study was aimed at elucidating as to what extent, in CHP-100 cells, C2-Cer shares the cytotoxic properties and metabolic characteristics of its related compound.

2. Materials and methods

2.1. Materials

CHP-100 human neuroepithelioma cells were obtained through the courtesy of Prof. G. Melino (University of Rome 'Tor Vergata'). Materials for cell culture, D-erythro-C2-Cer, D-erythro-C6-Cer, natural ceramide from bovine brain, glucosylceramide, Hoechst 33342, propidium iodide (PI), caspase-9 inhibitor N-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (Z-LEHD.fmk), caspase-8 inhibitor Z-Ile-Glu-Thr-Asp(OMe)-CH2F (Z-IETD.fmk), and ordinary bench reagents were from Sigma–Aldrich Chemical Co. Diacylglycerol kinase from E. coli was from Calbiochem Novachem Co. Silica gel 60 high-performance thin layer chromatography (HPTLC) plates were from Merck. The anti-poly(ADP)ribose polymerase (PARP) mouse monoclonal antibody was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). The chemiluminescence ECL detection system and [γ-^{32}P]ATP were from Amersham Corp.

2.2. Cell culture and treatments

CHP-100 cells were grown at 37 °C in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2. C6-Cer and C2-Cer were administered to cells in the culture medium from dimethylsulfoxide stock solutions (30 mM for each compound). After treatments for the indicated periods, cells were mechanically removed from the substrate directly in the culture medium and pelleted at 600 × g for 5 min at 4 °C. Cells were then washed with phosphate-buffered saline (PBS, pH 7.4) prior to further processing.

2.3. Apoptosis evaluation

Apoptosis was monitored by evaluation of the hypodiploid nuclei percent by flow cytometric analysis, after PI staining, as previously reported [14,15]. Briefly, cells were detached from the plates by trypsin treatment and centrifuged at 600 × g for 5 min; pellets were washed in PBS, placed on ice and overlaid with 0.5 ml of a solution containing 50 μg/ml PI, 0.1% Triton X-100 and 0.1% sodium citrate. After gentle resuspension in this solution cells were left at 4 °C for 30 min at least, in the absence of light, before analysis. PI-stained nuclei were analyzed using a FACScan Flow Cytometer (Becton-Dickinson, CA); fluorescence was measured between 565 nm and 605 nm. The data were acquired and analyzed by the Lysis II program (Becton-Dickinson, CA). In some experiments cells were fixed in 4% paraformaldehyde in PBS and incubated for 15 min with a solution of 0.5 μg/ml Hoechst 33342 in PBS; nuclei were then visualized with a Zeiss fluorescence microscope: fragmented and/or condensed elements were scored as apoptotic [14].

For analysis of PARP cleavage, cells were lysed in 62 mM Tris–HCl, pH 6.8, containing 2% SDS, 2 mM EDTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. After sonication, aliquots of the lysates were saved for protein determination [16] and, after addition of 0.05% β-mercaptoethanol, samples were boiled for 10 min. Proteins from cell lysates (40 μg) were resolved by 10% SDS-PAGE, transferred onto nitrocellulose paper and probed by Western blot, as previously described in detail [14,15]. Immunoreactivity was revealed by an ECL detection kit, according to the manufacturer's instructions.

2.4. Lipid analysis

Lipids were extracted by the method of Bligh and Dyer [17] with minor modifications. Briefly, cells were mechanically detached from the substrate directly into the culture medium and sedimented by centrifugation at 600 × g for 5 min, at 4 °C. The supernatant was discarded, the cell pellet was resuspended in chloroform/methanol/water (1:2:0.8, by volume) and maintained for 30 min at room temperature under frequent vigorous vortexing. Samples were then centrifuged at 1000 × g for 10 min to separate a pellet (for protein determination) and a lipid-containing supernatant. The supernatant was carefully removed, enriched with 1 ml chloroform and 1 ml water, vigorously vortexed and centrifuged at 600 × g for 5 min, to separate a lower chloroformic phase and an upper methanol/water phase. The chloroformic phase was saved, whereas the methanol/water phase was re-extracted two times with 2 ml chloroform: chloroformic extracts were pooled. The Bligh and Dyer's chloroformic phase was then dried, lipids resuspended in 0.1 M methanolic KOH, subjected to mild alkaline hydrolysis for 1 h at 37 °C and re-extracted by the Bligh and Dyer's method, as reported above. LC-Cer was quantitated by the diacylglycerol kinase method, using known amounts of commercial LC-Cer and for calibration, as previously reported in detail [15]. Due to the fact that short-chain ceramides are not substrates as good as LC-Cer for the diacylglycerol kinase assay [18], comparative evaluation of the levels of C2-Cer and C6-Cer was performed after HPTLC separation in chloroform/acetic acid (9:1, by vol) and detection by charring with 3% cupric acetate in 8% phosphoric acid [11]. Short-chain ceramide bands were then quantitated by densitometric analysis, using a computerized image processing system (Gel-Pro Analyzer), referring to known amounts of lipid standards, also processed by the Bligh and Dyer's method, in parallel with experimental samples.

For HPTLC separation of neutral glycosphingolipids (GSLs), samples were developed in chloroform/methanol/water (65:25:4, v/v/v). For GSLs detection, plates were sprayed with a solution made of 10.5 ml methanolic α-naphthol (5%, w/v), 6.5 ml concentrated sulfuric acid, 40.5 ml ethanol and 4 ml water, followed by heating for 10 min at 90 °C. Glucosylceramide (GlcCer) quantitation was performed by densitometric analysis, after HPTLC separation and spot visualization as described above, using a computerized image processing system (Gel-Pro Analyzer) and referring to known amounts of authentic lipid standard, developed along with samples [19]. Lipid amounts were referred to cell protein content. In some experiments, following HPTLC separation, simultaneous visualization of GSLs and short-chain ceramides was performed after charring, by dipping developed plates in a solution of 3% cupric acetate in 8% phosphoric acid and heating in an oven for 15 min at 180 °C [11].

2.5. Statistical analysis

Statistical significance was determined by paired Student's t test. Differences were considered significant at $P < 0.01$.

3. Results

3.1. Differential apoptotic effect of C2-Cer and C6-Cer in CHP-100 cells: evidence for a common intrinsic pathway

CHP-100 cells were incubated in growth medium enriched with a 30 μM concentration of either C2-Cer or C6-Cer and

apoptosis evaluated by 24 h. As assessed by FACS analysis, C6-Cer induced a potent cytotoxic response, with over a 40% of cells scored as apoptotic; on the other hand, cell viability was affected to a much lesser extent by C2-Cer, with only a rough 15% of apoptotic cells (Fig. 1A). The differential apoptotic response evoked by the two short-chain ceramides was confirmed by monitoring the extent of cleavage of the caspase substrate PARP (Fig. 1B) and by assessing the percentage of condensed and fragmented nuclei, after Hoechst-staining and microscopy analysis (Fig. 1C). Notably, Fig. 1C also shows that the cytotoxic effect elicited by both C2-Cer and C6-Cer was significantly reduced by the caspase-9 inhibitor z-LEHD-fmk, but not by the caspase 8 inhibitor z-IEDV, thus suggesting that apoptosis induction by short-chain ceramides involves a common intrinsc, but not extrinsic, apoptotic pathway [20].

3.2. Evidence for differential intracellular accumulation and metabolic utilization of C2-Cer and C6-Cer

Under the above-reported experimental conditions, the intracellular accumulation kinetics profiles of the two short-chain ceramides were compared. Fig. 2A shows that the levels of both compounds steeply elevated within 2 h from administration: at this time, however, C2-Cer levels displayed a 40% reduction in comparison to C6-Cer. On prolonging the incubation period up to 24 h, C6-Cer levels maintained at a fair steady-state, albeit with a faint tendency to decrease at late times, whereas C2-Cer displayed a slight tendency to further elevate, albeit without attaining C6-Cer levels (Fig. 2A).

Short-chain ceramide-evoked accumulation of LC-Cer via the sphingosine salvage pathway was then analyzed. Fig. 2B and C shows that C6-Cer administration induced a marked increase of LC-Cer levels that elevated about six-fold over basal by 24 h; at variance, no statistically significant increase of LC-Cer levels was observed after C2-Cer administration.

Since CHP-100 cells actively metabolize both C6-Cer and LC-Cer generated via the salvage pathway to GlcCer [5], it was monitored whether the lower intracellular accumulation of C2-Cer, in comparison to C6-Cer, was accounted, at least partly, by a more pronounced metabolic removal via direct glucosylation. In line with previous observations [5], cell exposure for 24 h to C6-Cer evoked a marked accumulation of both C6-GlcCer and LC-GlcCer; moreover, in line with the notion that C2-Cer is not utilized for LC-Cer synthesis via the salvage pathway, no increase in LC-GlcCer levels was observed with respect to basal (Fig. 3A and B). In addition, neither accumulation of C2-GlcCer was detected throughout the 24-h-period examined (Fig. 3A and B).

3.3. The increase of C2-Cer concentration in the medium associates with elevation of its intracellular levels and potentiation of apoptosis, in the absence of LC-Cer accumulation

To assess the existence of any causal relationship between the lower profile of C2-Cer intracellular accumulation and its attenuated apoptotic effect, as compared to C6-Cer, the former compound was administered to cells at increasing concentrations in the culture medium and its intracellular accumulation, as well as the apoptotic response, evaluated. As shown in Fig. 4, increasing C2-Cer

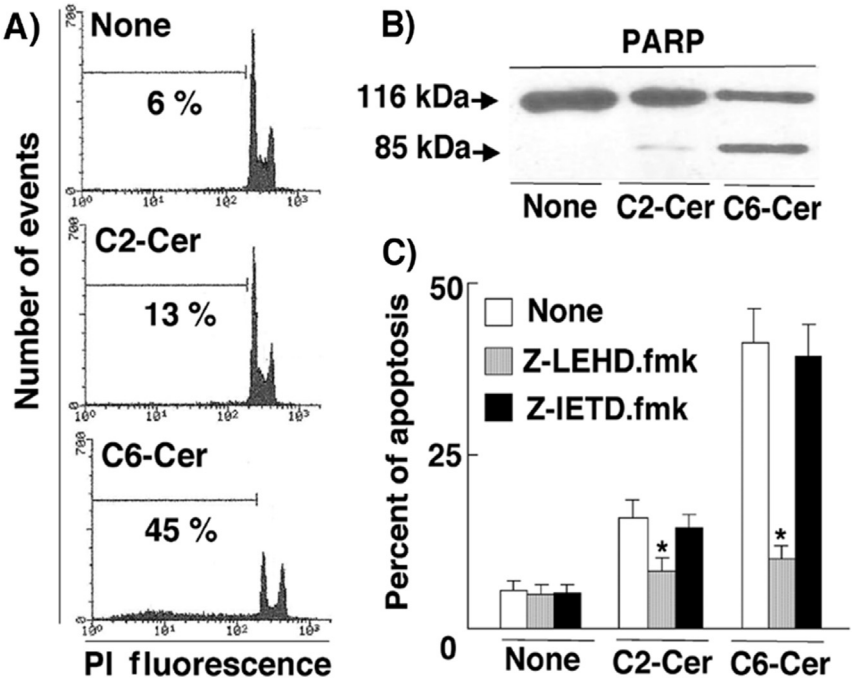

Fig. 1. Differential apoptotic effect of C2-Cer and C6-Cer in CHP-100 cells: evidence for a common intrinsic pathway. A) Cells were incubated for 24 h in complete growth medium in the presence of vehicle (none) or 30 μM C2-Cer or 30 μM C6-Cer for 24 h. At the indicated times cells adherent to the substrate as well as floating cells were collected, washed with PBS and apoptosis monitored by evaluation of the 'hypodiploid' nuclei percent (Ap) by flow cytometric analysis, as reported in the text. Results show a representative experiment repeated three times, with similar results. Percentages of apoptosis are reported under the bar covering the 'hypodiploid 'region. B) Cells were incubated for 24 h in the presence of vehicle alone (none), or 30 μM C6-Cer or 30 μM C2-Cer. Cells were then washed, lysed, and equal amounts of protein from total lysates were resolved by SDS-PAGE and probed for the native (116 kDa) and the cleaved form (85 kDa) of PARP. The figure is representative of three analyses performed with similar results. C) Three sets of cells (three dishes each) were preincubated for 30 min with vehicle (DMSO) or the caspase 9 inhibitor Z-LEHD.fmk (200 μM) or the caspase 8 inhibitor Z-IETD.fmk (200 μM). Samples from each set were then treated for additional 24 h with vehicle or 30 μM C2-Cer or 30 μM C6-Cer. Floating and attached cells from each sample were then collected and apoptosis evaluated by nuclei microscopy analysis, after staining with Hoechst 33342. Data are means ± SD of four experiments. Statistical significance: *P < 0.01, as from Student's t-test, as compared to samples treated with C6-Cer alone.

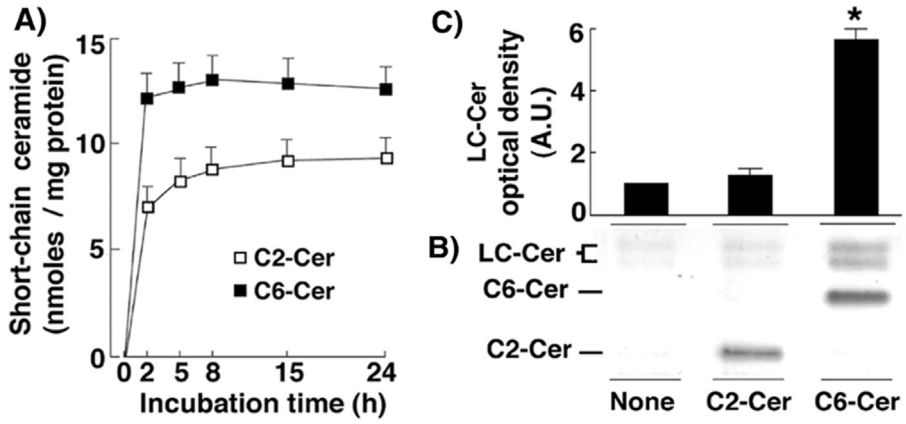

Fig. 2. Differential profiles of C2-Cer and C6-Cer intracellular accumulation and evidence that C2-Cer is not processed via the sphingosine salvage pathway. A) CHP-100 cells were incubated with vehicle (none) or 30 μM C2-Cer or 30 μM C6-Cer for the indicated times: thereafter, aliquots of the lower phase of the Bligh and Dyer's extracts from the different samples, corresponding to equal amounts of protein, were resolved by HPTLC and LC-Cer, C2-Cer and C6-Cer levels analyzed as reported in the text. Data are means ± SD of four independent experiments. B,C) CHP-100 cells were incubated with vehicle (none) or 30 μM C2-Cer or 30 μM C6-Cer for 24 h. Lipids were then extracted, ceramides resolved by HPTLC, visualized by charring (B) and LC-Cer levels compared by densitometric analysis (C). Results in panels C are expressed in arbitrary units (A.U.) and are means ± SD of four independent experiments.

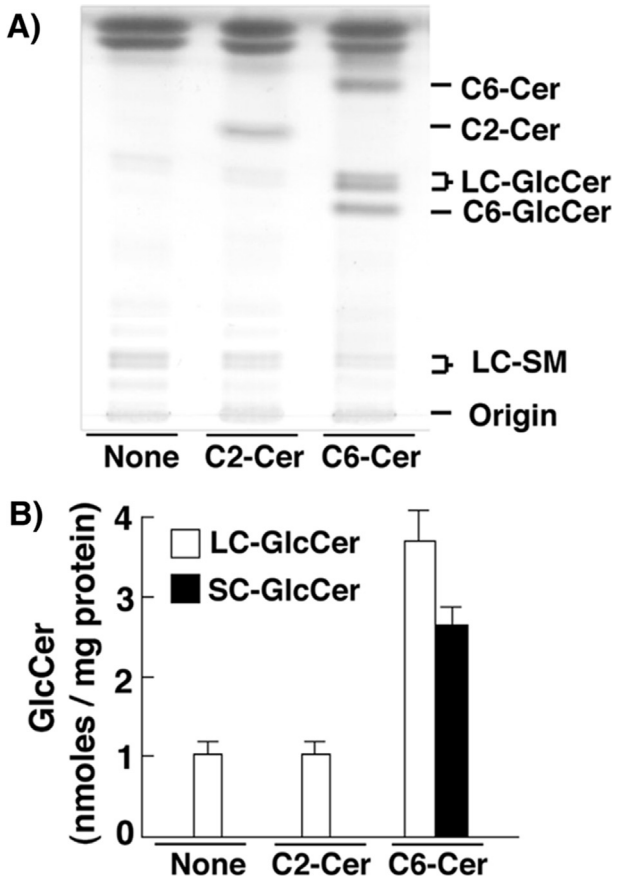

Fig. 3. C6-Cer, but not C2-Cer, induces intracellular accumulation of short- and long-chain GlcCer. CHP-100 cells were incubated in growth medium enriched with 30 μM C2-Cer or 30 μM C6-Cer for 24h. Cells were then harvested, lipids extracted and GlcCer separated by HPTLC. A) Representative HPTLC analysis, after lipid visualization of total lipids by charring. B) Histogram reporting the levels of short- and long-chain GlcCer in untreated cells, as well as in cells exposed to C2-Cer or C6-Cer. GlcCer was quantitated as described in the text and its levels referred to cell protein content. Data are means ± SD of four different experiments.

concentration in the medium to 45 μM or 60 μM resulted in a progressive increase of either its intracellular levels and of its cytotoxic effect. Remarkably, however, C2-Cer-induced LC-Cer accumulation was not yet observed under the above-reported experimental conditions.

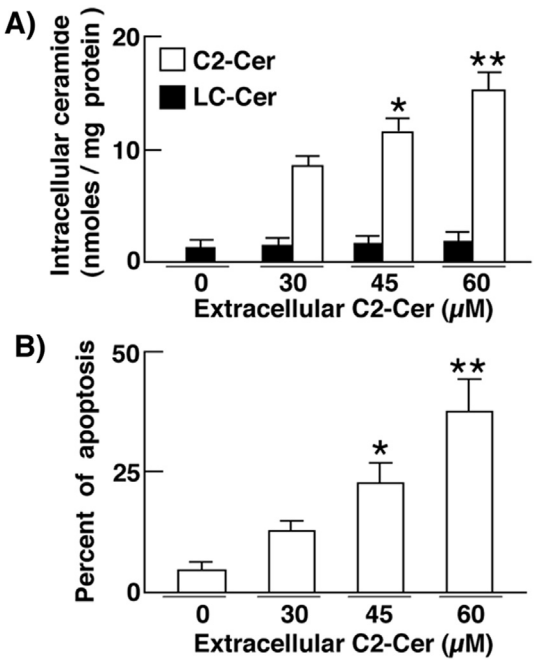

Fig. 4. Increasing C2-Cer concentration in the culture medium dose-dependently associates with enhancement of its intracellular levels and apoptosis, in the absence of LC-Cer accumulation. A) CHP-100 cells were incubated in culture medium enriched with C2-Cer at the indicated concentrations. After 24 h cells adherent to the substrate as well as floating cells were collected, washed in PBS and C2-Cer as well LC-Cer levels evaluated as reported in the text. Data are means ± SD of four experiments. Statistical significance: *P < 0.01, as compared to samples exposed to 30 μM C2-Cer, and **P < 0.01, as compared to samples exposed to 45 μM C2-Cer, after Student's t test. B) CHP-100 cells were incubated in culture medium enriched with C2-Cer at the indicated concentrations. After 24 h cells adherent to the substrate as well as floating cells were collected, washed in PBS and apoptosis monitored by evaluation of the 'hypodiploid' nuclei percent by flow cytometric analysis. Data are means ± SD of four experiments. Statistical significance: *P < 0.01, as compared to samples exposed to 30 μM C2-Cer, and **P < 0.01, as compared to samples exposed to 45 μM C2-Cer, after Student's t test.

4. Discussion

The above reported results demonstrate that C2-Cer and C6-Cer markedly differ in their apoptotic properties when administered at equimolar concentrations, within the low micromolar range, to CHP-100 cells. Moreover, the two synthetic ceramides displayed different metabolic characteristics: in fact, whereas C6-Cer was largely converted to LC-Cer via the sphingosine salvage pathway or directly glucosylated, C2-Cer was not appreciably utilized along the above-mentioned metabolic routes, under the experimental conditions herein employed.

Studies on different cell models pointed to the existence of a causal relationship between the scarce apoptotic effect of C2-Cer and the evidence that this compound is not metabolized to LC-Cer via the sphingosine salvage pathway [10,21]: this notion, however, does not apply to CHP-100 cells, since in this cell system it was previously shown that suppression of C6-Cer-evoked LC-Cer accumulation, by co-administration of the ceramide synthase inhibitor fumonisin B1, does not affect the apoptotic response [11]. Consistently, the body of evidence herein reported strongly indicates that, in CHP-100 cells, a major factor driving the differential cytotoxic effect of C2-Cer and C6-Cer resides in their differential intracellular accumulation profiles, with C6-Cer levels maintaining significantly higher than C2-Cer throughout the time-course investigated. To support this notion, it has been presently shown that, upon increasing C2-Cer concentration in the medium, both the intracellular levels of this compound and the apoptotic response were markedly increased, in the absence of any increase of LC-Cer levels. Notably, in line with this view, studies on other neural cell systems demonstrated that apoptosis induction requires administration of short-chain ceramides above threshold concentrations, whereas at lower concentrations these compounds may even be protective toward apoptosis induced by a variety of stimuli [22,23].

The reason as to why the recycling of short-chain ceramide to long-chain derivatives is necessary for apoptosis induction in certain cell systems but not in others is presently unclear; in this respect, however, it is worth recalling that whereas LC-Cer is able to segregate at the plasma membrane to form platforms that may be instrumental to clustering of death receptors, leading to apoptosis by activation of caspase 8, short-chain ceramides do not form separate phases [24,25]. On this basis, one could envisage the possibility that short-chain ceramide processing via the salvage pathway might, at least preferentially, associate with the extrinsic apoptotic pathway [20]. Consistently with this view, C6-Cer recycling to C16-Cer was shown to reverse resistance to 'tumor-necrosis factor-related apoptosis-inducing ligand' (TRAIL) in SW620 colon cancer cells [26]; moreover, in K562 leukemia cells, it was demonstrated that C6-Cer, but not C2-Cer, induces apoptosis via caspase 8 activation and it was envisaged that the phenomenon might be related to the differential properties of the two synthetic ceramides to be converted to long-chain derivatives [21].

Since short-chain ceramides are currently under trial in the perspective of therapeutic utilization [1–4], the present results may underscore the potential advantage of the use of C6-Cer over C2-Cer, either in the case that apoptosis occurs via the intrinsic or via the extrinsic apoptotic pathway. In the former case, in fact, notwithstanding its partial metabolic clearance through direct glucosylation, as well as through processing via the salvage pathway and subsequent glucosylation, its intracellular cellular levels held higher than C2-Cer that shows to be substantially metabolically inert, at least within the low micromolar range. Moreover, enhancement of C6-Cer intracellular levels can be further achieved by co-administration of GCS inhibitors [5]. In the case of apoptosis occurring via the extrinsic pathway C6-Cer may readily be converted to LC-Cer at concentrations relevantly lower than occurring for C2-Cer, thus possibly favoring the formation of platforms necessary for apoptosis induction [25].

Conflict of interest

None.

Acknowledgments

This work was supported by MIUR (Ministero dell'Istruzione dell'Università e della Ricerca) funds to Angelo Spinedi.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.107.

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