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Potentiation of okadaic acid-induced ceramide elevation but not apoptosis by inhibition of glucosylceramide synthase in human neuroepithelioma cells

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Abstract

Caspase-dependent apoptosis induced by okadaic acid (OA) in CHP-100 neuroepithelioma cells has previously been shown to associate with a rapid and sustained elevation in intracellular ceramide concentration. We now report that treatment of CHP-100 cells with OA also evoked a rapid elevation in glucosylceramide levels that was maintained at steady state as cells underwent apoptosis; moreover, as observed for ceramide, OA-induced glucosylceramide accumulation was not blocked by fumonisin B_1. Remarkably, when cell death was prevented by caspase inhibition, glucosylceramide accumulation was potentiated and ceramide elevation reduced, thus suggesting that, during apoptosis completion, accumulation of ceramide was partly driven by impairment of its glucosylation through a caspase-dependent mechanism. We studied whether ceramide glucosylation provided a mechanism for negative modulation of OA-induced apoptosis. We observed that the blocking of glucosylceramide synthesis markedly potentiated OA-induced ceramide elevation, but neither accelerated apoptosis onset nor potentiated the apoptotic response. These results indicate that modulation of ceramide glucosylation does not affect the apoptotic response to okadaic acid and suggest that caution must be exercised concerning the possibility that ceramide plays a key role in apoptosis induction.

Keywords: Ceramide; Glucosylceramide; Apoptosis; Protein phosphatase; Okadaic acid; Neurotumor

1. Introduction

There is general agreement that ceramide (*N*-acyl-erythro-sphingosine, Cer) is a key mediator in apoptosis induced by a variety of stimuli, including receptor ligation, irradiation, and drug treatment, on a wide range of cell systems [1,2]. In this respect, unequivocal evidence has been obtained by studies showing that stimuli evoking both programmed cell death and ceramide elevation lose their apoptotic potential after blocking of the metabolic pathway that leads to Cer generation [3–5]. In other studies, the role of Cer as a mediator of apoptosis has been largely inferred on the basis of the evidence that programmed cell death associates with intracellular Cer elevation and can be reproduced by administration of exogenous and cell-permeant short-chain Cer [6].

We have reported that OA, a potent inhibitor of serine–threonine PP produced by the marine sponge *Halichondria okadai* [7], induces apoptosis in CHP-100 human neuroepithelioma cells in a manner that largely involves caspase activation [8]; in addition, caspase-dependent apoptosis associates with an elevation in intracellular Cer levels [8]. These results, together with the finding that administration of short-chain Cer induces caspase-dependent apoptosis in CHP-100 cells [9], suggested that Cer might be the mediator linking sustained PP inhibition to apoptosis. CHP-100 cells actively glucosylate both endogenous and exogenously administered Cer [10]. In line with the notion that glucosylation provides a major pathway by which the apoptogenic pools of Cer are down-regulated [11–14], we have reported that, in CHP-100 cells, the apoptotic effect of short-chain Cer is potentiated by inhibition of GlcCer synthase [10]. We investigated here the kinetics of GlcCer accumulation, as observed in CHP-100 cells in relationship to OA-induced

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Abbreviations: OA, okadaic acid; PP, protein phosphatase; Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin; PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; and Z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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apoptosis. Moreover, we studied whether the apoptotic response triggered by OA is propagated by inhibition of Cer glucosylation.

2. Materials and methods

2.1. Materials

Material for cell culture was from GIBCO BRL. PDMP was from Calbiochem-Novachem. Fumonisin B_1 as well as Cer, GlcCer, and SM standards were from Sigma Chemical Co. The caspase inhibitor Z-VAD.fmk was from Alexis Co. [^{14}C]Palmitic acid (55.3 mCi/mmol) was from Amersham Corp. High-performance TLC silica gel 60 plates were from Merck.

2.2. Cell culture and apoptosis evaluation

CHP-100 cells were grown at 37° in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin, in a humidified atmosphere with 5% (v/v) CO_2. Cell treatments were performed in complete growth medium. Apoptosis was monitored by evaluation of the hypodiploid cell population by flow cytometric analysis. Cells were detached from the plates by trypsin treatment and centrifuged at 300 $\times$ *g* for 5 min; pellets were washed with PBS (pH 7.4), placed on ice, and overlaid with 0.5 mL of a solution containing 50 μg/mL of propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. After gentle resuspension in this solution, cells were left at 4° for at least 30 min, in the absence of light, before analysis. Propidium iodide-stained cells were analyzed using a FACScan Flow Cytometer (Becton Dickinson); fluorescence was measured between 565 and 605 nm. The data were acquired and analyzed by the Lysis II program (Becton Dickinson).

2.3. Cell labeling with [^{14}C]palmitate, lipid extraction, and separation

Cells grown in 35-mm plates were labeled with [^{14}C]palmitic acid (1 μCi/mL) for 24 hr. After washing with RPMI plus 0.1% BSA, cells were reincubated for 24 hr in label-free growth medium prior to any treatment. Lipids were extracted according to the method of Bligh and Dyer [15] and subjected to mild alkaline hydrolysis with 0.1 M methanolic KOH for 1 hr at 37°. After re-extraction, the chloroformic phase was analyzed by high-performance TLC. Monodimensional Cer and GlcCer separation was achieved by developing samples in chloroform/acetic acid (9:1, v/v) and chloroform/methanol/water (65:25:4, v/v), respectively. Two-dimensional SM resolution was achieved by developing samples in chloroform/methanol/25% NH_4OH/water (65:35:4:4, v/v) (first dimension) and in chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v) (second dimension). Lipid spots were visualized under iodine and scraped off from the plates into counting vials for radioactivity determination. Protein determination was carried out according to Lowry *et al.* [16].

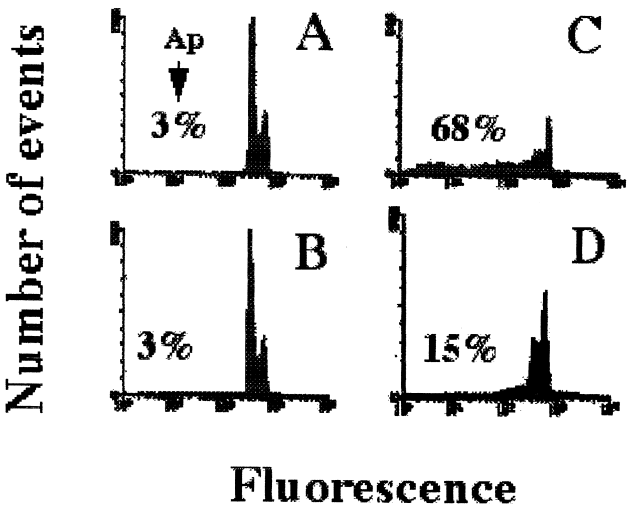

Fig. 1. Time-course of OA-induced apoptosis and the rescuing effect of Z-VAD.fmk. CHP-100 cells were either left untreated (A), incubated with 50 nM OA for 5 (B) or 24 hr (C), or incubated for 24 hr with 50 nM OA in the presence of 100 μM Z-VAD.fmk (D). After staining with propidium iodide, cells were analyzed by flow cytometry. Percentages of the hypodiploid (apoptotic, "Ap") cell populations are given.

3. Results

Fig. 1 shows the flow cytometric analysis of CHP-100 cells exposed for different times to 50 nM OA. In keeping with previous observations [8], apoptosis was not detected within 5 hr after addition of the PP inhibitor (panels A and B), but became extensive by 24 hr of treatment (panel C); moreover, cell death was largely blocked by the caspase inhibitor Z-VAD.fmk, 100 μM (panel D). We studied the temporal profile of the changes occurring in Cer and GlcCer levels upon exposure of CHP-100 cells to 50 nM OA, under conditions in which apoptosis was either allowed to occur or was inhibited by 100 μM Z-VAD.fmk. Fig. 2 shows that within 5 hr after OA addition, i.e. before apoptosis onset, not only Cer, but also GlcCer levels were already increased (panels A and B, respectively); moreover, at this time, changes in the levels of the two sphingolipids were not affected by the presence of the caspase inhibitor. On the other hand, as exposure to OA was prolonged beyond 5 hr, the accumulation profiles of Cer and GlcCer were markedly affected by the presence of Z-VAD.fmk: the inhibitor, in fact, blocked any further elevation in Cer levels and elicited a more pronounced accumulation of GlcCer (Fig. 2, panels A and B).

We have reported that OA-induced Cer accumulation is

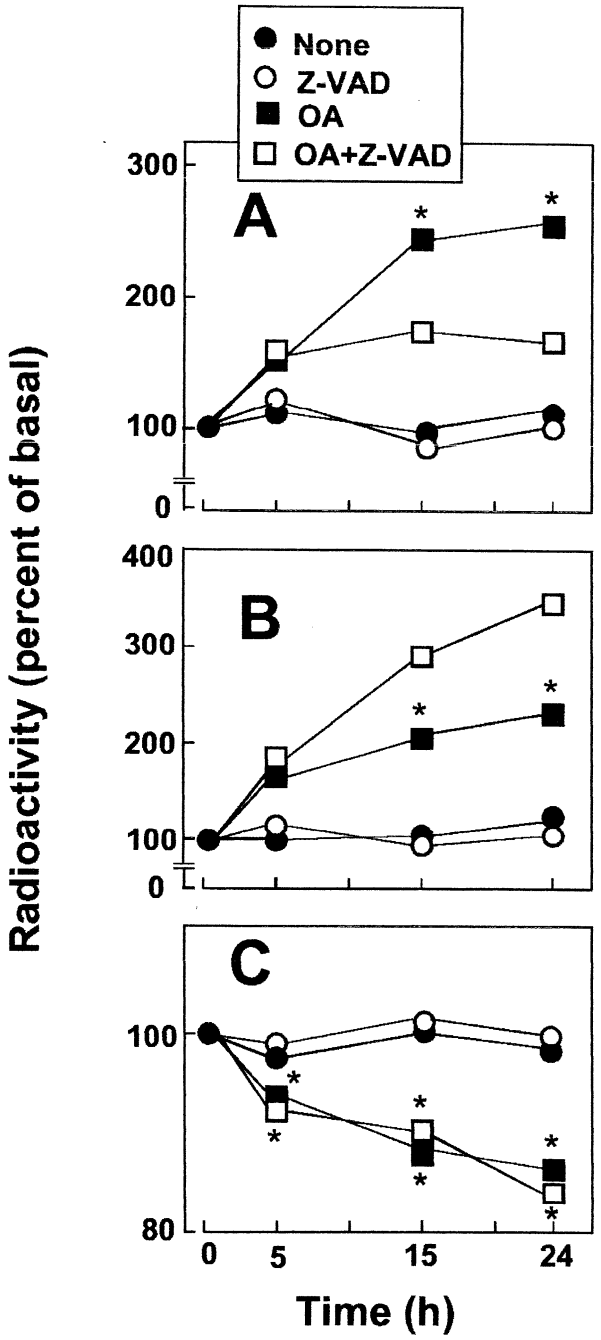

Fig. 2. Z-VAD.fmk effects on Cer, GlcCer, and SM levels in OA-treated cells. [^{14}C]Palmitate-labeled cells were incubated for the indicated times in the absence (circles) or presence (squares) of 50 nM OA, either in the absence (black symbols) or presence (white symbols) of 100 μM Z-VAD.fmk. Lipids were then extracted, resolved by high-performance TLC, and radioactivity in Cer (A), GlcCer (B), and SM (C) determined. Basal radioactivity (dpm/mg protein ± SD) was: 1400 ± 100 for Cer, 1050 ± 180 for GlcCer, and 28,510 ± 2,650 for SM. Results are means of four different experiments. Standard deviations (not shown) were within 15% of mean values. Statistical significance: $*P < 0.01$, as from paired data analysis, as follows. Panels A and B: samples treated with OA alone versus samples treated with OA plus Z-VAD.fmk. Panel C: samples treated with OA versus untreated with OA.

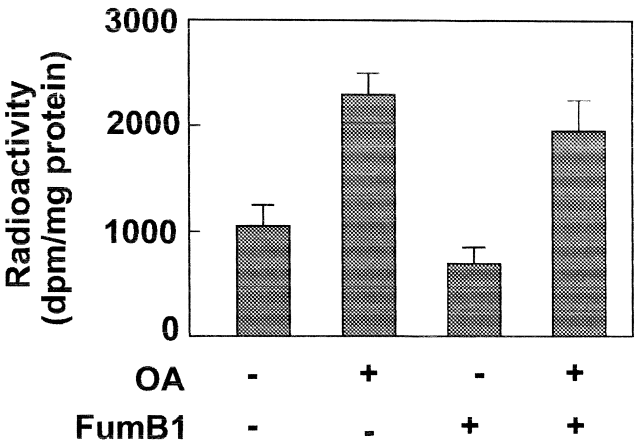

Fig. 3. Fumonisin B_1 does not suppress OA-evoked GlcCer accumulation. [^{14}C]Palmitate-labeled cells were incubated for 24 hr in medium containing 50 nM OA or vehicle, either in the presence or absence of 25 μM fumonisin B_1 (FumB1). Lipids were extracted, GlcCer isolated, and its radioactivity content measured. The presence or absence of OA or fumonisin B_1 is indicated by "+" and "−," respectively. Results are means ± SD of four different experiments. Differences between values referring to samples treated with OA alone or OA plus fumonisin B_1 were not statistically significant, as from paired data analysis.

not suppressed by the Cer synthase inhibitor fumonisin B_1 [8]. We now show in Fig. 3 that fumonisin B_1 did not even suppress OA-induced GlcCer accumulation, thus ruling out the possibility that glucosylation targets a separate neosynthesized Cer pool. In line with the possibility that both Cer and GlcCer elevation might follow SM hydrolysis, Fig. 2C shows that SM levels were decreased after cell exposure to OA; moreover, Z-VAD.fmk did not reverse the decrease in SM levels in OA-treated cells, thus clearly indicating that the phenomenon is upstream of caspase activation.

The above-reported results indicated that, during apoptosis completion, Cer accumulation was partly sustained by impairment of its conversion to GlcCer through a caspase-dependent mechanism. Since Cer may be upstream of caspase activation [9], we entertained the possibility that the initial rapid Cer glucosylation could partly account for the delay observed in apoptosis induction and that later reduction of GlcCer synthesis might provide a positive loop for amplification of the apoptotic response. On this basis, we questioned whether artificial impairment of Cer glucosylation could accelerate apoptosis onset and/or potentiate the apoptotic response to OA.

To address this issue, we monitored the effects of the GlcCer synthase inhibitor PDMP [17] on OA-induced Cer elevation and apoptosis. PDMP was used at 30 μM: at this concentration, the compound was reported to be non-toxic for CHP-100 cells when administered alone, but to enhance short-chain Cer-induced apoptosis by potently inhibiting its glucosylation [10]. Unexpectedly, we found that PDMP did not affect the kinetics or the magnitude of the apoptotic response induced by 50 nM OA (Fig. 4A). This result prompted us to investigate whether, upon blocking of glu-

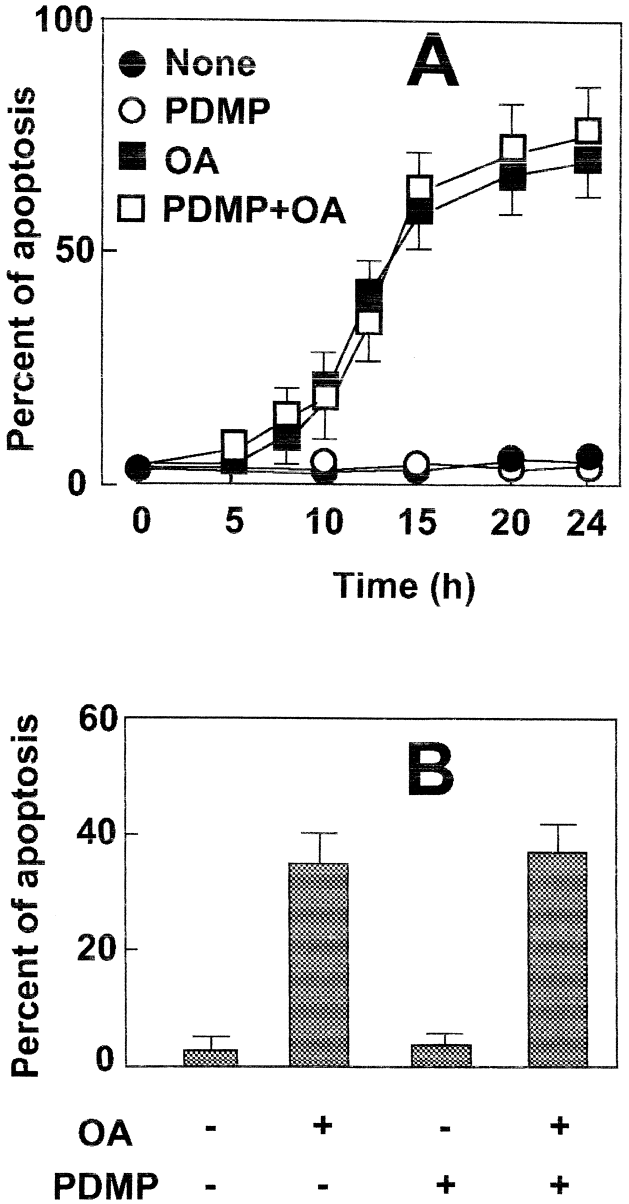

Fig. 4. PDMP does not propagate OA-induced apoptosis. (A) Cells were incubated for the indicated times in the absence (circles) or presence (squares) of 50 nM OA, either in the absence (black symbols) or presence (white symbols) of 30 μM PDMP. (B) Cells were either left untreated or treated for 24 hr with 20 nM OA, 30 μM PDMP, or 20 nM OA plus 30 μM PDMP. Cells were harvested and processed for apoptosis determination by flow cytometry. In each panel, results are means ± SD of four different experiments. Where not shown, SDs fall inside the symbol. The presence of PDMP did not significantly affect basal or OA-induced apoptosis, as from paired data analysis.

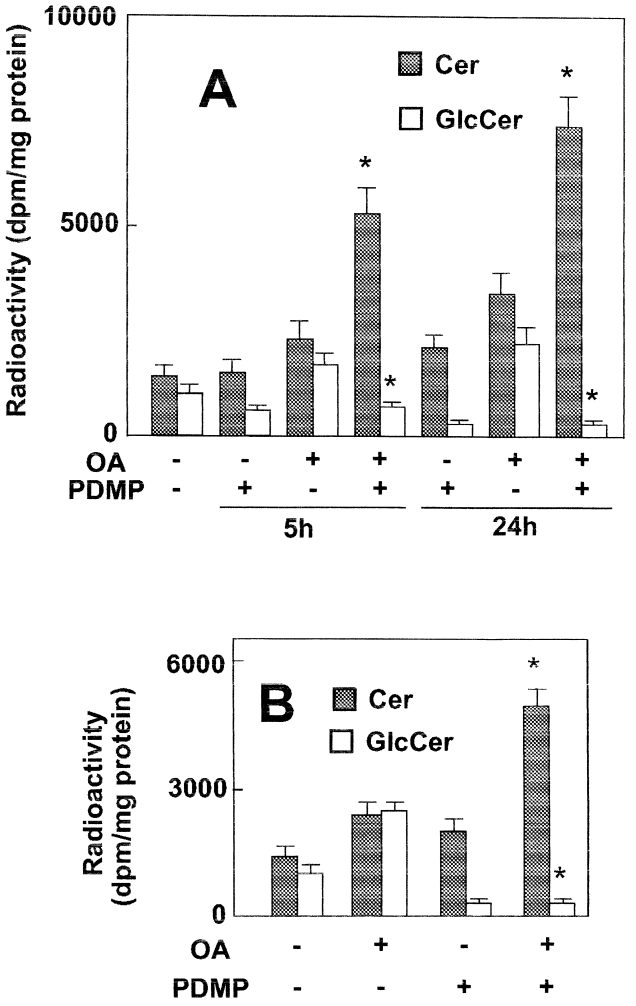

Fig. 5. PDMP enhances OA-induced Cer accummulation. (A) [^{14}C]Palmitate-labeled cells were either left untreated or treated for 5 or 24 hr with 50 nM OA, 30 μM PDMP, or 50 nM OA plus 30 μM PDMP. Cer and GlcCer were then isolated and their radioactivity measured. (B) [^{14}C]Palmitate-labeled cells were either left untreated or treated for 24 hr with 20 nM OA, 30 μM PDMP, or 20 nM OA plus 30 μM PDMP. Cer and GlcCer were then isolated and their radioactivity measured. The presence or absence of OA or PDMP is indicated by "+" and "−", respectively. In each panel, data are means ± SD of four different experiments. Statistical significance: $^*P < 0.01$, as from paired data analysis of samples treated with OA plus PDMP versus samples treated with OA alone.

cosylation, Cer was down-regulated through alternative metabolic pathways. To this aim, we monitored the effects of PDMP on OA-induced Cer elevation at 5 or 24 hr, i.e. before apoptosis onset or when the phenomenon had reached its maximal extent. As shown in Fig. 5A, PDMP not only blocked GlcCer synthesis, but, as observed at both times, potentiated Cer accumulation approximately three-fold in comparison with samples treated with OA alone.

The effects of PDMP on OA-induced ceramide accumulation and apoptosis were also studied using the PP inhibitor at a 20-nM concentration, i.e. the lowest one at which OA was found to induce caspase-dependent apoptosis [8]. Most remarkably, the GlcCer to Cer ratio was found to peak at this concentration when monitored after 24-hr cell incubation with increasing OA doses (not shown, but compare data from Fig. 5A and 5B). On this basis, one could expect that the moderate apoptotic response produced by 20 nM OA could be accounted for by a reduced Cer accumulation, due to sustained lipid removal through glucosylation. As shown

in Fig. 4B, apoptosis induced by a 24-hr treatment of CHP-100 cells with 20 nM OA was not affected by the presence of 30 μM PDMP; conversely, the GlcCer synthase inhibitor potentiated OA-induced Cer elevation by more than 300% (Fig. 5B).

4. Discussion

GlcCer synthesis is not only the first step in the synthesis of complex glycosphingolipids, but has also been implicated in intracellular Cer homeostasis. Indeed, a substantial body of evidence now points to an inverse relationship between tumor cell ability to sustain pronounced GlcCer synthesis and sensitivity to those agents that induce apoptosis through the Cer pathway [11–14]. Moreover, it has been demonstrated that inhibition of GlcCer synthase potentiates short-chain Cer-induced apoptosis [10]. In this study, we have shown that Cer produced in response to CHP-100 cell exposure to OA was rapidly converted to GlcCer in the period during which cell viability was fully maintained, but, as apoptosis started to occur, Cer glucosylation markedly declined through a caspase-dependent mechanism. This observation led us to investigate whether the initial rapid removal of Cer by glucosylation was related to apoptosis delay and whether the negative modulation of GlcCer synthesis by caspase activation provided a mechanism for apoptosis amplification. We have found that potentiation of OA-induced Cer accumulation, caused by the blocking of lipid removal through the GlcCer synthase inhibitor PDMP, did not affect the apoptotic response in CHP-100 cells. Thus, modulation of Cer glucosylation seems to be dissociated from the apoptotic response. One possible explanation for our results is that Cer produced after OA treatment requires compartmentalization to be effective and, differently from other systems, loses its apoptogenic properties once cycled to the site of glucosylation (i.e. the Golgi network). Alternatively, one might envisage that OA evokes the generation of both apoptogenic and non-apoptogenic Cer pools in distinct cell compartments, and that only the latter are selectively targeted for glucosylation. Compartmentalization could reconcile the discrepancy between the present results and the previous observation that apoptosis induced by *N*-hexanoylsphingosine in CHP-100 cells is potentiated by GlcCer synthase inhibition [10]. In fact, owing to its short fatty acid moiety, synthetic Cer is expected to interact more loosely with the membrane environment and to partition more easily in the aqueous phase than its naturally occurring counterparts. A consequence ensuing from this characteristic is that short-chain Cer concentration in the various membrane compartments may be mainly driven by physical equilibrium rather than by membrane flow; thus, the blocking of short-chain Cer glucosylation may in turn lead to elevation of its concentration in all membrane compartments, including those involved in the apoptotic response. Nevertheless, our results also raise the question as to whether the rapid and sustained Cer elevation evoked by OA is indeed involved in apoptosis induction or is rather a metabolic response to the stress caused by PP inhibition. With respect to this point, it is worth mentioning that OA, administered to intact cells at the concentrations presently employed, is expected to predominantly inhibit PP type 2A [18], and that Cer is a potent PP type 2A activator [19]. Thus, the dysregulated phosphorylation state of one or more critical protein(s) could provide a sufficient condition for activation of the apoptotic program, without any intervening role of Cer.

The role of Cer in apoptosis has recently become a matter of debate [20–23]. In those cases in which apoptosis associates with accumulation of neosynthesized Cer, the use of the Cer synthase inhibitor fumonisin B_1 has proved crucial to confirm or dismiss the apoptogenic role of the lipid [3,24]. On the other hand, when Cer is generated through SM hydrolysis, blocking of the reaction is difficult to achieve, due to poor availability of specific and non-toxic sphingomyelinase inhibitors. In these cases, the apoptotic role of Cer is inferred, *bona fide*, on the ability of exogenously administered Cer to reproduce the apoptotic outcome. However, the question has been posed as to whether short-chain and endogenous Cer share the same biological properties [21,25]. Indeed, the data provided herein and results previously reported by our group [10] indicate that blocking of glucosylation of short-chain Cer and of endogenous Cer generated in response to OA induce different apoptotic outcomes in CHP-100 cells. This implies that caution must be exercised concerning the possibility that Cer produced after cell treatment with OA plays a key role in apoptosis induction.

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References

[1] Mathias S, Peña LA, Kolesnick RN. Signal transduction of stress via ceramide. Biochem J 1998;335:465–80.
[2] Hannun YA, Luberto C. Ceramide in the eukaryotic stress response. Trends Cell Biol 2000;10:73–80.
[3] Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. Cell 1995; 82:405–14.
[4] Lehtonen JY, Horiuchi M, Daviet L, Akishita M, Dzau VJ. Activation of the *de novo* biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis. J Biol Chem 1999;274:16901–6.
[5] Lin T, Genestier L, Pinkoski MJ, Castro A, Nicholas S, Mogil R, Paris F, Fuks Z, Schuchman EH, Kolesnick RN, Green DR. Role of acidic sphingomyelinase in Fas/CD95-mediated cell death. J Biol Chem 2000;275:8657–63.

[6] Hannun YA. Functions of ceramide in coordinating cellular responses to stress. Science 1996;274:1855–9.
[7] Wera S, Hemmings BA. Serine/threonine protein phosphatases. Biochem J 1995;311:17–29.
[8] Spinedi A, Di Bartolomeo S, Di Sano F, Rodolfo C, Ambrosino A, Piacentini M. Ceramide accumulation precedes caspase-dependent apoptosis in CHP-100 neuroepithelioma cells exposed to the protein phosphatase inhibitor okadaic acid. Cell Death Differ 1999;6:618–23.
[9] Spinedi A, Amendola A, Di Bartolomeo S, Piacentini M. Ceramide-induced apoptosis is mediated by caspase activation independently from retinoblastoma protein post-translational modification. Biochem Biophys Res Commun 1998;243:852–7.
[10] Spinedi A, Di Bartolomeo S, Piacentini M. Apoptosis induced by *N*-hexanoylsphingosine in CHP-100 cells associates with accumulation of endogenous ceramide and is potentiated by inhibition of glucocerebroside synthesis. Cell Death Differ 1998;5:785–91.
[11] Lavie Y, Cao H, Volner A, Lucci A, Han T-Y, Geffen V, Giuliano AE, Cabot MC. Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. J Biol Chem 1997;272:1682–7.
[12] Liu Y-Y, Han T-Y, Giuliano AE, Cabot MC. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. J Biol Chem 1999;274:1140–6.
[13] Liu Y-Y, Han T-Y, Giuliano AE, Ichikawa S, Hirabayashi Y, Cabot MC. Glycosylation of ceramide potentiates cellular resistance to tumor necrosis factor-alpha-induced apoptosis. Exp Cell Res 1999;252:464–70.
[14] Sietsma H, Veldman RJ, Kolk D, Ausema B, Nijhof W, Kamps W, Vellenga E, Kok JW. 1-phenyl-2-decanoylamino-3-morpholino-1-propanol chemosensitizes neuroblastoma cells for taxol and vincristine. Clin Cancer Res 2000;6:942–8.
[15] Bligh EC, Dyer WJ. A rapid method for total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7.
[16] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[17] Shayman JA, Lee L, Abe A, Shu L. Inhibitors of glucosylceramide synthase. Methods Enzymol 2000;311:373–87.
[18] Millward TA, Zolnierowicz S, Hemmings BA. Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 1999;24:186–91.
[19] Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. Ceramide activates heterotrimeric protein phosphatase 2A. J Biol Chem 1993;268:15523–30.
[20] Watts JD, Gu M, Polverino AJ, Patterson SD, Aebersold R. Fas-induced apoptosis of T cells occurs independently of ceramide generation. Proc Natl Acad Sci USA 1997;94:7292–6.
[21] Hofmann K, Dixit VM. Ceramide in apoptosis—does it really matter? Trends Biochem Sci 1998;23:374–7.
[22] Watts JD, Gu M, Patterson SD, Aebersold R, Polverino AJ. On the complexities of ceramide changes in cells undergoing apoptosis: lack of evidence for a second messenger function in apoptotic induction. Cell Death Differ 1999;6:105–14.
[23] Karasavvas N, Zakeri Z. Relationships of apoptotic signaling mediated by ceramide and TNF-alpha in U937 cells. Cell Death Differ 1999;6:115–23.
[24] Di Bartolomeo S, Di Sano F, Piacentini M, Spinedi A. Apoptosis induced by doxorubicin in neurotumor cells is divorced from drug effects on ceramide accumulation and may involve cell cycle-specific caspase activation. J Neurochem 2000;75:532–9.
[25] Tepper AD, de Vries E, van Blitterswijk WJ, Borst J. Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. J Clin Invest 1999;103:971–8.