Ceramide accumulation precedes caspase-dependent apoptosis in CHP-100 neuroepithelioma cells exposed to the protein phosphatase inhibitor okadaic acid

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Abstract

The protein phosphatase inhibitor okadaic acid (OA) dose-dependently induced apoptosis in CHP-100 neuroepithelioma cells when administered for 24 h at concentrations ranging from 10 – 100 nM. Apoptosis was largely, albeit not completely, dependent on cystein protease (caspase) activation. CPP32 processing and poly(ADP-ribose) polymerase (PARP) cleavage started to be observed only at 20 nM OA; moreover, the caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD.fmk) (100 μM) had negligible effect on apoptosis induced by 10 nM OA, but rescued from death an increasing cell fraction as OA concentration was raised from 20 – 100 nM. Cell treatment for 24 h with OA induced ceramide accumulation; the phenomenon started to be evident at 20 nM OA and reached its maximum at 50 – 100 nM OA. In cells exposed to 50 nM OA, ceramide was already elevated by 5 h; at this time, however, PARP cleavage and apoptosis were not yet observed. Z-VAD.fmk (100 μM) had no effect on ceramide elevation induced by 50 nM OA within 5 h, but markedly reduced ceramide accumulation as the incubation was prolonged to 24 h. The latter phenomenon was accompanied by elevation of glucosylceramide levels, thus suggesting that a caspase-dependent reduction of glucosylceramide synthesis might contribute to late ceramide accumulation. Short-chain ceramide (30 μM) induced apoptosis in CHP-100 cells and its effect was additive with that evoked by OA (10 – 20 nM). These results suggest that ceramide generation might be an important mechanism through which sustained protein phosphatase inhibition induces caspase activation and apoptosis in CHP-100 cells.

Keywords: apoptosis; okadaic acid; ceramide; caspases; neuroepithelioma

Introduction

Sustained inhibition of serine-threonine phosphatases (PPs) has been shown to induce apoptosis in a variety of cell systems.[1–7] There is evidence that multiple pathways activated by PP inhibition may converge to elicit the apoptotic response. In various instances, apoptosis induced by okadaic acid (OA), a potent inhibitor of PP type 2A and PP type 1,[8] has been shown to associate with cell cycle deregulation leading to abortive mitosis,[2,7,9] a view consistent with the notion that PPs can modulate the expression and/or the activity of various proteins that ensure proper progression through the replicative cycle.[8,10] PP inhibition, on the other hand, has also been shown to modulate the expression of proteins involved in apoptosis control. In HL60 cells, for instance, OA-induced apoptosis is preceded by down-regulation of the anti-apoptotic protein Bcl-2, due to destabilization of its transcript;[11] moreover, in MCF-7 and lens epithelial cells, OA-induced apoptosis associates with enhanced expression of p53 and bax, two pro-apoptotic genes.[3,6] A role for cystein proteases (caspases) in apoptosis induced by sustained PP inhibition has also been reported; in particular, prolonged exposure of ML-1 leukemia cells to the PP inhibitor calyculin A has been shown to result in caspase activation and cell death.[12] Ceramide (N-acyl-erythro-sphingosine) is a lipid mediator generated by neosynthesis or sphingomyelin hydrolysis in response to cell exposure to a variety of stimuli, including receptor ligation, drug treatment, irradiation and environmental stress (reviewed by Hannun[13] and Mathias et al.[14]). A large body of evidence suggests that ceramide generation is a key event leading to apoptosis through mechanisms that may involve caspase activation;[13,14] in this respect, we have previously demonstrated that administration of short-chain ceramide induces caspase-dependent apoptosis in CHP-100 human neuroepithelioma cells.[15] Presently, it is unknown whether sustained PP inhibition leads to ceramide generation. Herein we report that apoptosis induced by OA in CHP-100 cells is largely, albeit not completely, dependent on caspase activation and that ceramide accumulation is selectively associated with caspase-dependent apoptosis.

Results

OA-induced apoptosis is largely but not entirely caspase-mediated

CHP-100 cells exposed for 24 h to nanomolar concentrations of OA underwent rounding, detachment from the substrate and, eventually, apoptosis. As assessed by flow cytometric analysis, the appearance of a hypodiploid population started to be observed around 10 nM OA and was fairly maximal at 50–100 nM OA (Figure 1); apoptosis occurrence was confirmed by Hoechst staining, showing outstanding chromatin condensation and progressive enhancement of nuclear fragmentation as the inhibitor concentration was raised from 10–100 nM (not shown). The possible involvement of caspase activation in apoptosis was initially studied by monitoring processing of the CPP32 proenzyme and cleavage of the endogenous caspase substrate poly (ADP-ribose) polymerase (PARP),[16] in cells incubated for 24 h with different OA concentrations. As shown in Figure 2 (upper panel), substantial accumulation of CPP32 low MW fragments started to be observed in cells treated with 20 nM OA; in the presence of 100 nM OA CPP32 processing was fairly maximal, as indicated by the marked downregulation of the 32 kDa proenzyme. PARP cleavage fairly followed the kinetics of CPP32 processing; as shown in Figure 2 (lower panel), accumulation of the 85 kDa PARP cleavage product started to be observed at 20 nM OA, whereas incubation with 100 nM OA led to disappearance of the 116 kDa native form of the protein. We further investigated whether caspase inhibition resulted in prevention of OA-induced apoptosis; this issue was of special relevance for cell death evoked by 10 nM OA, since, at this concentration, neither PARP cleavage nor CPP32 processing were observed. Figure 1 shows that the caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (Z.VAD.fmk) (50 μM) had negligible effect in preventing apoptosis induced by cell incubation for 24 h with 10 nM

OA; however, as OA concentration was raised above 10 nM, an increasing cell fraction was rescued from death. Notably, increasing Z-VAD.fmk concentration to 100 μM had no further effect on apoptosis prevention.

OA evokes ceramide accumulation: insensitivity to fumonisin B$_1$ and relationship to apoptosis and caspase activation

We investigated whether OA treatment induced elevation of endogenous ceramide levels in the cell system under study. We have previously reported that in unstimulated CHP-100 cells ceramide mass amounts to about 3 pmol/nmol of lipid phosphate and that changes of ceramide mass can reliably be monitored by radiometric analysis, after cell labeling to equilibrium with [^{14}C]-palmitate.[17] Therefore, [^{14}C]-palmitate-labeled cells were exposed for 24 h to different OA concentrations and radioactivity accumulation in ceramide monitored. Figure 3 shows that OA started to induce ceramide elevation at concentrations higher than 10 nM; at 50–100 nM OA, when apoptosis was maximal, ceramide levels reached 230–250% of those observed in untreated cells. Incidentally, after administration of 10 nM OA, ceramide accumulation was not detected even at times shorter than 24 h. Fumonisin B$_1$, a fungal toxin that inhibits ceramide synthase has been successfully used to block ceramide elevation due to neosynthesis and to assess the relevance of the phenomen-
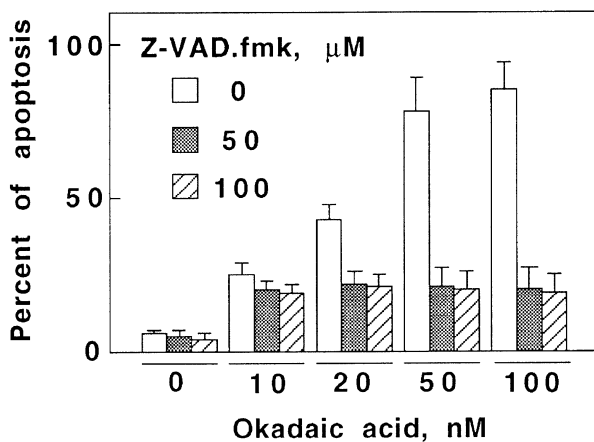

Figure 1 Apoptosis induced by OA in CHP-100 cells is largely, but not completely, dependent on caspase activation. CHP-100 cells were preincubated for 1 h with vehicle or Z-VAD.fmk (50 μM or 100 μM) prior to addition of OA at the indicated concentrations. After 24 h cells were harvested and processed for apoptosis determination by flow cytometric analysis, as described in the text. Results are means ± S.D. of four different experiments
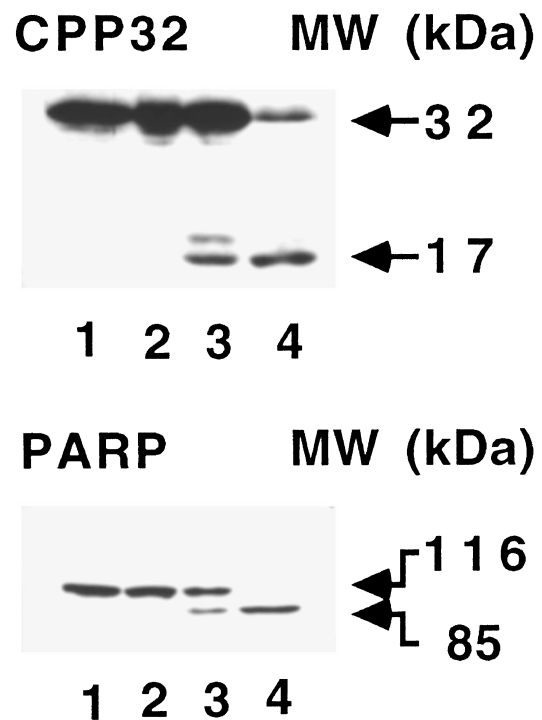

Figure 2 OA induces CPP32 processing and PARP cleavage. Cells were incubated for 24 h with vehicle (lane 1) or OA at the concentration of 10 nM (lane 2), 20 nM (lane 3) or 100 nM (lane 4). Samples were then lysed and analyzed by Western blotting for CPP32 (upper panel) or PARP (lower panel), as described in the text

on in apoptosis induction.[18–20] Figure 4 shows that fumonisin B_1 (25 μM), administered for 24 h to [^{14}C]-palmitate-labeled CHP-100 cells slightly reduced basal ceramide levels; however, when the toxin was administered in combination with 50 nM OA, it was ineffective in suppressing ceramide elevation. Similar results were obtained when fumonisin B_1 concentration was raised to 50 μM; moreover, fumonisin B_1 had no effect on OA-induced apoptosis (not shown). It has been reported that sphingomyelinase-mediated ceramide
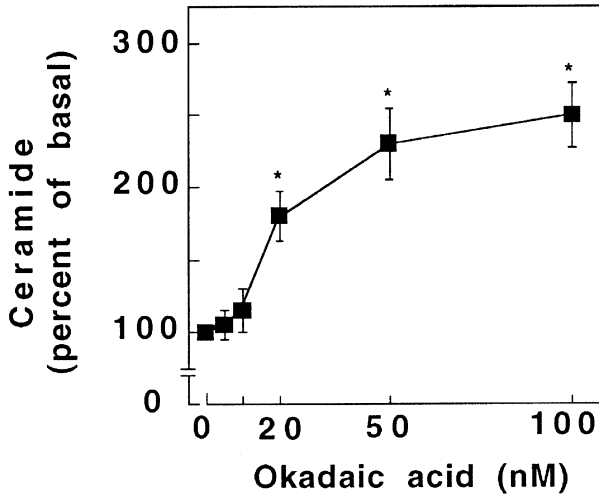
generation may occur after activation of upstream caspases;[21,22] to address this point in our system, CHP-100 cells were exposed to 50 nM OA and the kinetics of apoptosis induction, caspase activation and ceramide accumulation investigated. Figure 5 (upper panel) shows that the onset of apoptosis occurred after 5 h from OA administration and the phenomenon was fairly maximal by 15 h; consistently with this result, caspase activation, as monitored by PARP cleavage, was not observed within 5 h (Figure 5, lower panel). Remarkably, the time-course of ceramide accumulation displayed a different profile; in fact, as shown in Figure 5 (upper panel), ceramide was substantially elevated already within 5 h from OA administration, namely prior to caspase activation and apoptosis onset. In keeping with the above mentioned results, ceramide elevation, as observed after 5 h of cell treatment with 50 nM OA, was not affected by 100 μM Z-VAD (not shown); however, upon prolonging cell treatment with 50 nM OA to 24 h, ceramide accumulation was reduced by a rough 50% by the presence of 100 μM Z-VAD (Figure 6). Notably, Figure 6 shows that cell treatment for 24 h with 50 nM OA increased glucosylceramide levels, thus indicating that, after production, ceramide partly undergoes metabolism

Figure 3 OA evokes ceramide accumulation. CHP-100 cells were labeled to equilibrium with [^{14}C]-palmitate (1 μCi/ml) and then exposed to vehicle or OA at the indicated concentrations for 24 h. Lipids were then extracted, subjected to mild alkaline hydrolysis, resolved by HPTLC as reported in the text and ceramide radioactivity determined. Results are means $\pm$ S.D. of four different experiments. Statistical significance: *$P<0.01$, as compared to samples treated with carrier only, as from paired data analysis
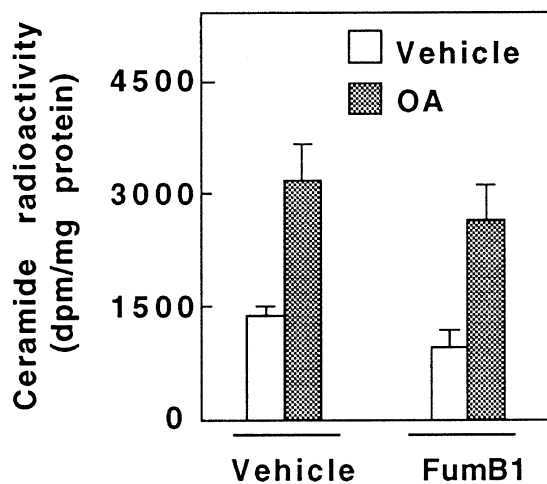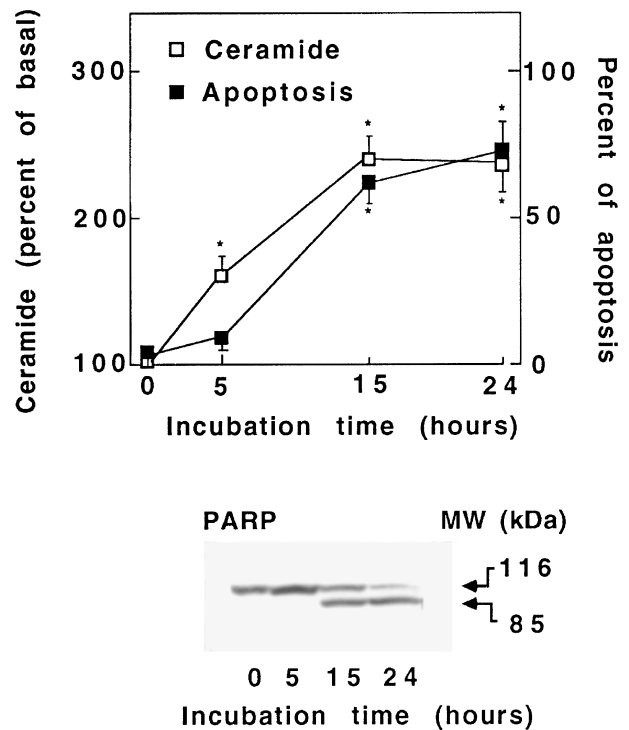
Figure 4 OA-evoked ceramide accumulation is not suppressed by fumonisin B1. [^{14}C]-Palmitate-labeled cells were incubated for 24 h with vehicle or 50 nM OA, either in the absence (Vehicle) or presence of 25 μM fumonisin B_1 (FumB1). Lipids were then extracted, subjected to mild alkaline hydrolysis, resolved by HPTLC as reported in the text and ceramide radioactivity determined. Results are means $\pm$ S.D. of four different experiments

Figure 5 OA-induced ceramide accumulation precedes caspase activation and apoptosis. (Upper panel) [^{14}C]-Palmitate-labeled cells were incubated for the indicated times with vehicle or 50 nM OA. Lipids were then extracted, subjected to mild alkaline hydrolysis, resolved by HPTLC as reported in the text and ceramide radioactivity determined. Treatment with 50 nM OA for the indicated times was also performed on unlabeled cells that were then processed for apoptosis determination by flow cytometric analysis. Results are means $\pm$ S.D. of four different experiments. Statistical significance: *$P<0.01$, as compared to samples treated with carrier only, as from paired data analysis. (Lower panel) Cells were incubated for the indicated times with vehicle or 50 nM OA and total lysates analyzed by Western blotting for PARP
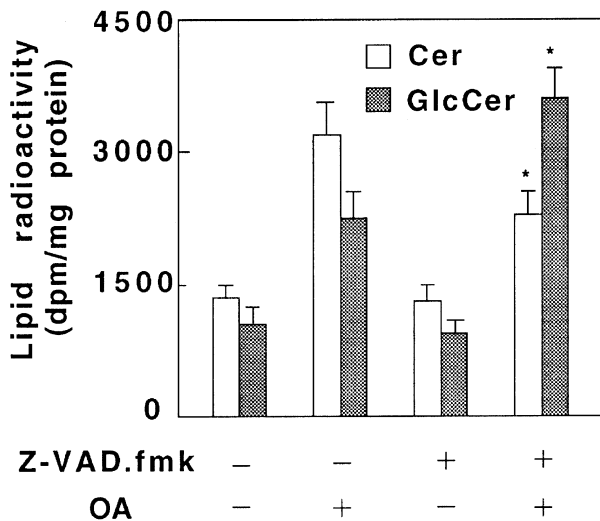
Figure 6 Z-VAD.fmk inhibits late ceramide accumulation evoked by OA but increases accumulation of glucosylceramide. [^{14}C]-Palmitate-labeled cells were incubated for 24 h with vehicle or 50 nM OA, either in the presence or absence of 100 μM Z-VAD.fmk. Lipids were then extracted, subjected to mild alkaline hydrolysis resolved by HPTLC as reported in the text and radioactivity in ceramide (Cer) and glucosylceramide (GlcCer) determined. Presence or absence of OA or Z-VAD.fmk in the incubation media are indicated by '+' or '−', respectively. Results are means ± S.D. of four different experiments. Statistical significance: *$P < 0.01$, as from paired data analysis

Figure 7 OA does not prevent short-chain ceramide-induced apoptosis. CHP-100 cells were incubated for 24 h with vehicle or 30 μM C$_6$-Cer, either in the absence or presence of OA at the indicated concentrations. Apoptosis was determined by flow cytometric analysis. Results are means ± S.D. of four different experiments. Statistical significance: *$P < 0.01$, as compared to samples treated with OA only, as from paired data analysis

through glucosylation; most remarkably, combined treatment with 50 nM OA and 100 μM Z-VAD.fmk did not impair but, indeed, elevated glucosylceramide levels (Figure 6).

OA, at concentrations eliciting cell death, does not block the apoptotic effect of short-chain ceramide

It is well established that ceramide activates a PP2A,[23,24] whose role in apoptosis is, however, ill-defined. Since PP2A are strongly inhibited by OA (Ki=0.2 nM)[8] and it has been mentioned that OA can prevent ceramide-induced apoptosis,[25] we were prompted to investigate whether, in CHP-100 cells, the combined treatment with N-hexanoyl sphingosine (C$_6$-Cer) and OA induced an apoptotic response higher or equal to that elicited by OA alone; in the latter case, in fact, any role for ceramide as a possible mediator of apoptosis induced by OA had to be dismissed. In the study, OA was employed at the concentrations of 10 or 20 nM; these concentrations, while expected to inhibit PP2A, did not cause *per se* extensive apoptosis, thus allowing evaluation of the contribution given to cell death by C$_6$-Cer. As shown in Figure 7, OA did not inhibit C$_6$-Cer-induced apoptosis; in fact, when cells were incubated for 24 h in media containing 30 μM C$_6$-Cer plus the PP inhibitor at the above-mentioned concentrations, the apoptotic response was fairly the sum of that elicited by short-chain ceramide and OA alone.

Discussion

We have reported that apoptosis induced by the PP inhibitor OA in CHP-100 cells is largely, albeit not completely, dependent on caspase activation; in addition, we have
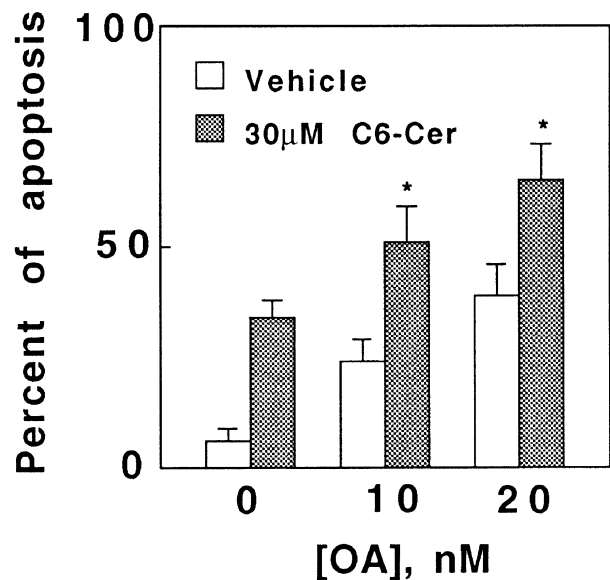
shown that OA-induced and caspase-mediated apoptosis associates with elevation of intracellular ceramide levels. Ceramide has been suggested to provide a key lipid mediator in apoptosis, largely based on the evidence that various agents able to induce cell death also stimulate its accumulation and that administration of cell-permeant ceramides can reproduce the apoptotic response. Based on the observation that short-chain ceramide induces caspase-mediated apoptosis in CHP-100 cells,[15] results herein reported raise the possibility that ceramide elevation may be involved in mediating the apoptotic response elicited by sustained PP inhibition. The relationships between ceramide accumulation and caspase activation have turned to be complex. Recently, it has been reported that sphingomyelinase-mediated ceramide generation occurring after receptor stimulation may be under the control of upstream caspases;[21,22] in another study the role of ceramide in receptor-mediated apoptosis has been challenged and it has been suggested that ceramide accumulation is the consequence rather than the cause of activation of a death executioner caspase.[26] In our system, OA induced ceramide elevation was already observed before PARP cleavage and apoptosis onset; at this time, lipid accumulation was also insensitive to the caspase inhibitor Z-VAD.fmk. However, as apoptosis progressed to completion, ceramide elevation was largely sustained by a mechanism that was sensitive to Z.VAD.fmk. While these results rule out the possibility that ceramide accumulation is a mere consequence of cell death, the question arises as to the mechanism(s) that drive late accumulation of the active lipid. We can not exclude that late ceramide accumulation occurs, at least in part, due to a caspase-mediated sphingomyelinase activation; however, since in OA-treated cells Z-VAD.fmk also

elicited a marked elevation of glucosylceramide, the possibility holds that late ceramide accumulation might be sustained by block of its removal through glucosylation. This view may be in line with a recent report showing that caspase-dependent downregulation of glucosylceramide synthase activity largely contributes to the kinetics and magnitude of ceramide elevation in tumor necrosis factor stimulated rhabdomyosarcoma cells.[27] Moreover, it has been shown that the multidrug-resistant state of cancer cells may be associated with elevated glucosylceramide synthase activity and can be reversed by inhibition of ceramide glucosylation;[28–30] thus, suppression of ceramide glucosylation might relevantly contribute to apoptosis propagation. Surprisingly enough, evidence for caspase involvement in cell death induced by sustained PP inhibition has, to date, seldomly been provided. In this respect, Morana and coworkers[12] have reported that apoptosis induced by prolonged exposure of ML-1 leukemia cells to calyculin A associates with PARP cleavage; since apoptosis occurred in the setting of retinoblastoma protein shift to the hypophosphorylated state, the authors suggested that suppression of a retino-blastoma protein kinase activity could be involved in ICE/ CED3 protease activation. In a set of experiments that have not been shown we observed that retinoblastoma protein shift to the hypophosphorylated state (and protein downregulation, as well) also occurred in CHP-100 cells, in association with OA-evoked and caspase-mediated apoptosis. Intriguingly, retinoblastoma protein shift to the hypophosphorylated state was not observed in cells treated with 10 nM OA (not shown). Based on a previous report indicating that ceramide can elicit both retinoblastoma protein dephosphorylation and caspase activation,[15] our results suggest that OA-induced ceramide generation might be upstream of both phenomena. The mechanism through which OA elicits ceramide generation remains to be elucidated and might involve sphingomyelinase activation: in this respect, in fact, we have observed that fumonisin B_1, a potent inhibitor of ceramide neosynthesis was ineffective in blocking OA-induced ceramide accumulation and apoptosis. Moreover, since OA is a potent inhibitor of both PP2A and PP1[8] it remains to be established the contribution given by inhibition of the above mentioned PPS to ceramide generation. A result herein reported whose elucidation will also need further study is that OA, at the lower concentration tested (i.e. 10 nM), was able to induce apoptosis through a mechanism that was independent on ceramide production and caspase activation. Given the fact that control of protein phosphorylation provides a basic mechanism through which cell metabolism and functions are controlled, it is not unexpected that PP inhibitors might signal for apoptosis through multiple mechanisms that may depend on the pattern of the inhibited PPs and by the specific cell response.

Materials and Methods

Materials

CHP-100 human neuroepithelioma were obtained through the courtesy of Prof. G. Melino (University of Rome 'Tor Vergata', Italy). Material for cell culture was from Gibco BRL (MD, USA). OA and fumonisin B_1 were from Sigma Chemical Co. (St. Louis, MO, USA).

The anti-PARP mouse monoclonal antibody was from Biomol Res. Lab. (Plymouth Meeting, PA, USA); The anti-CPP32 rabbit polyclonal antibody was from Pharmingen (San Diego, CA, USA). Z-VAD.fmk was from Alexis Co. (San Diego, CA, USA). C_6-Cer was from Calbiochem Novachem Co. (La Jolla, CA, USA). [^{14}C]-Palmitic acid (55.3 mCi/mmol) and the chemiluminescence ECL detection system were from Amersham Corp. (Bucks., UK). High-performance thin layer chromatography (HPTLC) silica gel 60 plates were from Merck (Darmstadt, Germany).

Cell culture and apoptosis evaluation by flow cytometric analysis

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% (v/v) CO_2. Cell treatments were performed in complete growth medium. Apoptosis was monitored by evaluation of the pre-G_1 cell population, after 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 phosphate-buffered saline (pH 7.4), placed on ice and overlaid with 0.5 ml of a solution containing 50 μg/ml propidium iodide, 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. Propidium iodide-stained cells were analyzed using a FACScan Flow Cytometer (Beckton-Dickinson, CA, USA); fluorescence was measured between 565 and 605 nm. The data were acquired and analyzed by the Lysis II program (Becton-Dickinson, CA, USA).

Western blotting

Cells were washed with phosphate-buffered saline (pH 7.4) and 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 lysates were saved for protein determination[31] and, after addition of 0.05% β-mercaptoethanol, samples were boiled for 10 min. Proteins from cell lysates (40 μg) were resolved by SDS–PAGE, transferred overnight at 25 mA onto nitrocellulose paper and analyzed by Western blot as previously described.[32] ECL detection was performed according to the manufacturer's instructions.

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 h. After washing with RPMI plus 0.1% bovine serum albumin, cells were reincubated for 24 h in label free growth medium prior to any treatment. Lipids were extracted according to[33] and subjected to mild alkaline hydrolysis with 0.1 M methanolic KOH for 1 h at 30°C. After re-extraction, the chloroformic phase was analyzed by HPTLC. Ceramide and glucosylceramide separation was achieved by developing samples in n-hexane/diethylether/acetic acid (25 : 75 : 1, vol/vol) for full plate height, followed by development in the same direction in chloroform/methanol/0.7 N NH₄OH (20 : 5 :0.5, vol/vol), up to 3 cm from the top, and again in the same direction in n/hexane/ diethylether/acetic acid (25 : 75 : 1, vol/vol) for full plate height.[17] Lipids were visualized under iodine and spots scraped off from the plates into counting vials for radioactivity determination. Radiometric estimation of changes in ceramide levels was confirmed by gas-chromatographic analysis, as previously reported.[17]

Acknowledgements

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