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Ceramide induces apoptosis in PC12 cells

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Abstract The novel lipid second messenger, ceramide, induced apoptosis in PC12 cells as determined morphologically by nuclear appearance and internucleosomal DNA fragmentation. Apoptosis was induced by exogenous C_2-ceramide in a dose- and time-dependent manner. Natural ceramide and C_6-ceramide had a similar effect. This response was specific since the structural analog C_2-dihydroceramide and other related lipids failed to initiate apoptosis. The apoptotic effect of ceramide also depends critically on cell plating density. Furthermore, the peptide inhibitor of interleukin-1β converting enzyme (ICE)-like proteases, Z-VAD.FMK, completely prevented the nuclear changes induced by ceramide, implicating the involvement of ICE-like protease activation in ceramide-induced apoptosis in PC12 cells.

Key words: Ceramide; Apoptosis; PC12 cell; Interleukin-1β converting enzyme-like protease

1. Introduction

Apoptosis is an evolutionary conserved and distinct form of programmed cell death characterised by an array of morphological criteria which include chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blebbing [1,2]. Apoptosis is a critical feature of mammalian development. For instance, in the developing nervous system neurons are generated in excess, compete for limited supplies of survival factors and up to 50% of immature neurons are eliminated by apoptosis [3,4]. Moreover, it is now recognised that defects in apoptosis are associated with a number of neurodegenarative diseases [5].

Ceramide, the second messenger of the sphingomyelin pathway, has emerged as a pleotropic mediator that regulates cell cycle arrest, differentiation and apoptosis [6,7]. The generation of endogenous ceramide is regulated by such agonists as tumour necrosis factor-α, Fas ligand, interleukin-1, ionising radiation and chemotherapeutic drugs, but the exact mechanisms involved remain to be determined. There is an increasing amount of evidence implicating ceramide as an important second messenger in neuronal signalling pathways. Nerve growth factor activates the sphingomyelin cycle through the p75 neurotrophin receptor, a member of the tumour necrosis factor receptor family, resulting in differentiation in T9 glioma cells [8]. Likewise, ceramide is involved in the differentiation of neuroblastoma Neuro2a cells [9]. Contrary evidence suggests a role for p75 in the promotion of apoptosis [10–12]. Furthermore, it has recently been demonstrated that ceramide can induce apoptosis in primary cultures of rat mesencephalic neurons [13] and embryonic chick neurons [14].

The rat pheochromocytoma PC12 cell line has been extensively utilised as a model to study the mechanisms regulating neuronal survival and apoptotic death [15–17]. Previous reports have indicated that treatment of PC12 cells with either exogenous ceramide [18] or bacterial SMase [19] inhibits nerve growth factor-induced differentiation. To our knowledge, ceramide effects on apoptosis in these cells have not been reported.

In the present study we determined the effect of ceramide, added exogenously in the form of short-chain ceramide analogs and natural ceramide, on apoptosis in PC12 cells. Our results demonstrate that ceramide specifically induces apoptosis in PC12 cells and that the apoptotic response depends critically on cell plating density. Furthermore, the peptide inhibitor of ICE-like proteases, Z-VAD.FMK, completely prevented the nuclear changes induced by ceramide, implicating the involvement of this family of proteases in the terminal stages of apoptosis.

2. Materials and methods

2.1. Materials

N-acetylsphingosine (C_2-ceramide), *N*-acetylsphinganine (C_2-dihydroceramide), *N*-hexanoylsphingosine (C_6-ceramide) and 1,2-dioctanoyl-sn-glycerol (DAG) were all obtained from Biomol. Natural ceramide (type III) and sphingomyelinase (SMase) from *S. aureus* were purchased from Sigma. Arachidonic acid (AA) was from Cayman Chemical. Hoechst 33258 was from Calbiochem. Benzyloxycarbonyl–Val–Ala–Asp (OMe) fluoromethylketone (Z-VAD.FMK) was obtained from Enzyme Systems Products. Stock solutions of lipids were prepared in DMSO or ethanol, except natural ceramide (Cer) which was prepared in ethanol/dodecane (98:2, v/v), as described by Ji et al. [20].

2.2. Cell culture

The rat pheochromocytoma cell line, PC12, was provided by Dr. R. Rush (Flinders Medical Centre, Adelaide, SA). Stock cultures were maintained in DMEM (high glucose) supplemented with 10% horse serum, 5% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. For all experiments, PC12 cells were detached by trituration, resuspended in complete DMEM and plated on to poly-L-ornithine-coated plastic culture dishes for 18–24 h prior to further treatments.

2.3. Determination of apoptosis by fluorescence microscopy

Morphological determination of levels of apoptosis was performed by labelling the cells with the nuclear stain Hoechst 33258 and visualisation by fluorescence microscopy. Briefly, PC12 cells, pre-plated in 6-well plastic culture dishes (4×10^4 cells/well), were washed in serum-free DMEM and resuspended in DMEM (2% FCS) in the presence of short-chain ceramide analogs, natural Cer, DAG, AA, or SMase. Final concentrations of DMSO or ethanol did not exceed 0.2% and in the case of ethanol/dodecane did not exceed 0.198%/0.002%. Treatments were continued for the indicated times and cells were stained

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Abbreviations: C_2-ceramide, *N*-acetylsphingosine; C_2-dihydroceramide, *N*-acetylsphinganine; C_6-ceramide, *N*-hexanoylsphingosine; Cer, natural ceramide; SMase, sphingomyelinase; DAG, 1,2-dioctanoyl-sn-glycerol; AA, arachidonic acid; Z-VAD.FMK, benzyloxycarbonyl–Val–Ala–Asp fluoromethylketone; ICE, interleukin-1β converting enzyme

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with Hoechst 33258 (5 μg/ml). Nuclei (blue) that were condensed or fragmented were scored as apoptotic. In all cases solvent alone had no effect on the morphological appearance or viability of PC12 cells.

2.4. Quantitative cell death ELISA

PC12 cells (1.2×10^5/60 mm dish) were treated for 18 h, removed from the dish, centifuged (2000 rpm, 10 min) and washed once. Cytoplasmic fractions were prepared from lysates by centrifugation at $20\,000\times g$ for 10 min. The cell death detection ELISA (Boehringer Mannheim), which quantitatively determines levels of cytoplasmic histone-associated DNA fragments, was performed according to manufacturer's instructions and absorbances were measured at 405 nm.

2.5. Photomicrography

Cells were treated for 18 h, washed gently in Krebs–Ringer–Hepes buffer, and fixed in paraformaldehyde (4%). After fixation, nuclei were stained with Hoechst 33258 (5 μg/ml) and photomicrographs (×400 magnification) were taken using a Leica Dialux 20 EB microscope.

2.6. Analysis of internucleosomal DNA fragmentation

Internucleosomal DNA fragmentation was analysed by agarose gel electrophoresis according to the method of Gong et al. [21]. Briefly, PC12 cells (5×10^6) were removed from plates, collected by centrifugation and fixed in 70% ethanol. DNA was extracted with 0.2 M phosphate–citrate buffer (pH 7.8), incubated with 0.25% NP-40 and RNase A (1 mg/ml) at 37°C for 30 min and treated with proteinase K (1 mg/ml) at 37°C for a further 30 min. Samples were loaded on to a 2% agarose gel and electrophoresis was performed at 5 V/cm for 4 h. DNA in gels was visualised under UV light after staining with ethidium bromide (0.5 μg/ml).

3. Results and discussion

PC12 cells were plated at low density and subsequent treatments were in low serum-containing media (2% FCS), since it is well recognised that biologically active lipids, such as ceramide, can be sequestered by lipid-binding serum proteins [22]. In this defined system control PC12 cells remained attached to the culture substratum and viability was greater than 95% for at least 24 h. Addition of exogenous ceramide, in the form of the short-chain cell-permeable analog C_2-ceramide, to PC12 cells resulted in a number of morphological changes that are characteristic of apoptosis. These included cell shrinkage, detachment from the substratum, blebbing of the cell membrane and, ultimately, an increase in cell death. To examine whether these changes induced by exogenous ceramide were indeed apoptosis, cells were stained with Hoechst 33258. The nuclei of control PC12 cells stained uniformly with this dye, indicating that the nuclei were intact and the cells were viable (Fig. 1A). Conversely, treatment of cells with C_2-ceramide (20 μM) caused nuclear fragmentation and condensation (Fig. 1B) and increased the levels of apoptosis as determined quantitatively by nuclei staining with Hoechst 33258 (Fig. 2A) or by a DNA fragmentation ELISA (Fig. 2B). C_6-ceramide, a ceramide analog with a longer 6-carbon fatty acid chain, similarly induced apoptosis (Fig. 2A,B). However, this analog was maximally effective at a lower concentration (10 μM) and displayed greater cytotoxicity at higher concentrations. Recent observations have demonstrated that natural Cer can be delivered to cells using a solvent mixture of ethanol and dodecane [20]. Treatment of PC12 cells with natural Cer resulted in apoptosis (Fig. 2A,B). Indeed, the effect of natural Cer was extremely sensitive in this system, in that low concentrations of natural Cer (50 ng/ml) induced high levels of apoptosis within 8 h. The specificity of ceramide-

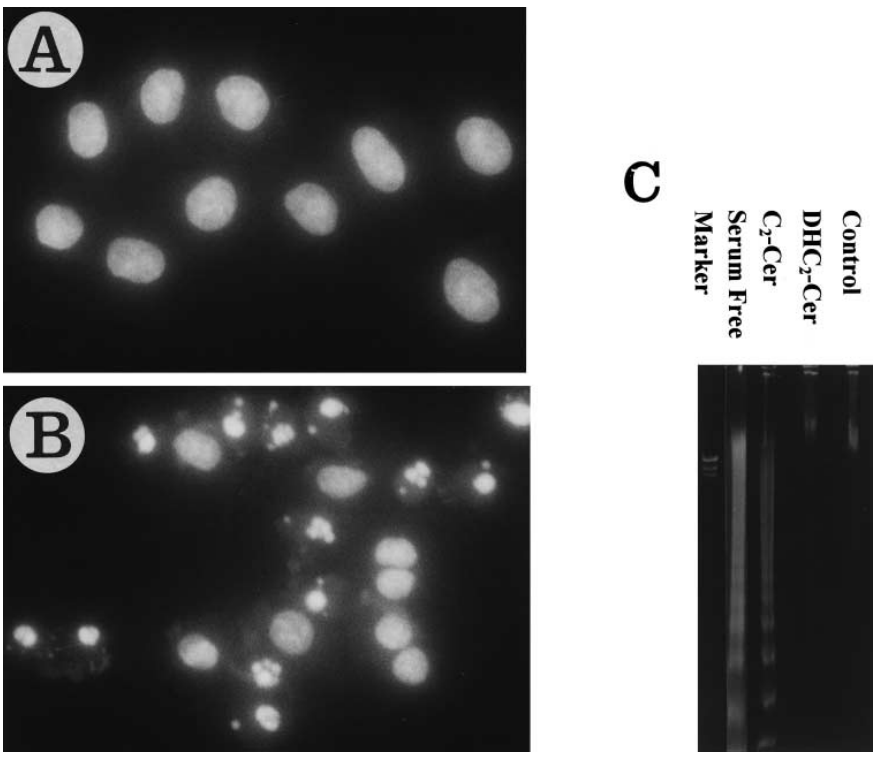

Fig. 1. Morphological appearance of PC12 cells treated with either vehicle alone (A) or 20 μM C_2-ceramide (B) for 20 h. Nuclei were stained with Hoechst 33258 and fluorescence photomicrographs (×400 magnification) were taken. In (C), internucleosomal DNA fragmentation was detected by agarose gel electrophoresis of DNA from cells treated with vehicle alone (Control), 20 μM C_2-dihydroceramide (DHC_2-Cer), 20 μM C_2-ceramide (C_2-Cer), or in the absence of serum (Serum free) for 24 h. The marker is *Hin*dIII digest of λ DNA.

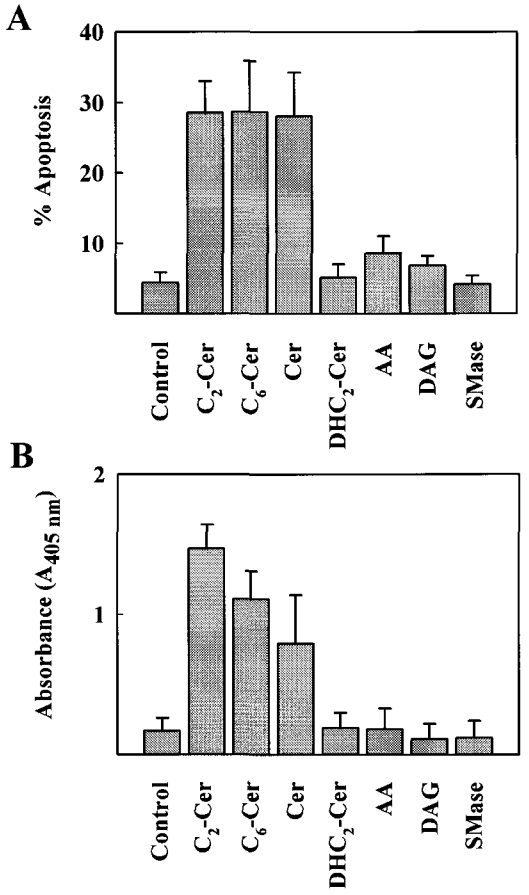

Fig. 2. Quantitative determination of apoptosis in PC12 cells. Levels of apoptosis were determined by (A) nuclei staining with Hoechst 33258 and (B) the cell death detection ELISA in PC12 cells treated in the absence (Control) or the presence of 20 μM C_2-ceramide (C_2-Cer), 10 μM C_6-ceramide (C_6-Cer), 20 μM C_2-dihydroceramide (DHC_2-Cer), 20 μM AA, 20 μM DAG, or 200 mU/ml SMase for 20 h, or 50 ng/ml natural ceramide (Cer) for 8 h. In each case the data represent means ± S.D. from at least four separate experiments.

induced apoptosis was investigated by determining the effects of structural analogs of ceramide and other related lipids. The ceramide analog C_2-dihydroceramide, which lacks the C4–5 *trans* double bond in the sphingolipid backbone that is necessary for the biological effects of ceramide [23], failed to induce a quantitative increase in the levels of apoptosis (Fig. 2A,B). In addition, equimolar concentrations (20 μM) of DAG and AA, structurally related lipid second messengers, failed to initiate apoptosis in PC12 cells (Fig. 2A,B). Further confirmation that ceramide induces apoptosis was evidenced by the characteristic pattern of internucleosomal DNA fragmentation detected by agarose gel electrophoresis of soluble cytoplasmic DNA (Fig. 1C). Indeed, treatment with C_2-ceramide produced a pattern of DNA cleavage similar to that obtained from PC12 cells subjected to serum starvation, a well-defined apoptotic trigger [15,16]. No DNA fragmentation was observed in either control cells or cells treated with C_2-dihydroceramide for 24 h.

Recent studies on the membrane localisation of sphingomyelin have identified two distinct pools that are defined by their sensitivity to hydrolysis by bacterial SMase [24]. The first pool is bacterial SMase-sensitive and is located in the outer leaflet of the plasma membrane, whereas the second pool is internal and resistant to bacterial SMase hydrolysis. In HL-60 cells, the signalling pool of sphingomyelin is bacterial SMase-insensitive [24]. Evidence also suggests that neurotrophins mobilise the same bacterial SMase-insensitive pool of sphingomyelin [18]. Treatment of PC12 cells with exogenous bacterial SMase failed to induce apoptosis (Fig. 2A,B), suggesting that in these cells the signalling pool of sphingomyelin does not reside in the outer leaflet of the plasma membrane. However, in the absence of a distinct agonist that stimulates the hydrolysis of sphingomyelin in PC12 cells it will be difficult to accurately determine the location of a putative signalling pool pertinent to apoptosis. Although sphingomyelin hydrolysis appears to account for the major source of ceramide, additional metabolic pathways may be important. Indeed, it has been suggested that drug-induced apoptosis involves the activation of ceramide synthase resulting in the de novo synthesis of ceramide [25].

The kinetics of ceramide-induced apoptosis were determined next. Exogenous C_2-ceramide (5–25 μM) was added to PC12 cells and levels of apoptosis were assessed after 4 h, 8 h, 16 h and 24 h (Fig. 3). Basal levels of apoptosis remained low (> 5%) in untreated cells. Little evidence of increased apoptosis could be detected with either 5 μM or 10 μM C_2-ceramide over 24 h. However, with 15 μM and 20 μM C_2-ceramide, apoptosis was markedly increased by 8 h (Fig. 3) and by 16 h, 23.2% and 28.1% of cells displayed apoptotic morphologies with 15 μM and 20 μM C_2-ceramide, respectively. Maximal levels of apoptosis were 27.6% and 28.6% with 15 μM and 20 μM C_2-ceramide, respectively, at 24 h. At higher concentrations (≥25 μM C_2-ceramide) the apoptotic effect of ceramide declined, but levels of cell death were elevated and displayed morphological characteristics of necrosis. Together, these data indicate that in PC12 cells ceramide induces a time and concentration dependent increase in apoptosis.

Previous work has established that the biological effects of exogenously added ceramide in cellular systems are subject to surface dilution kinetics [22]. Since PC12 cells were seeded at low cell density in our experiments, it was determined whether cell plating density influenced the apoptotic response induced

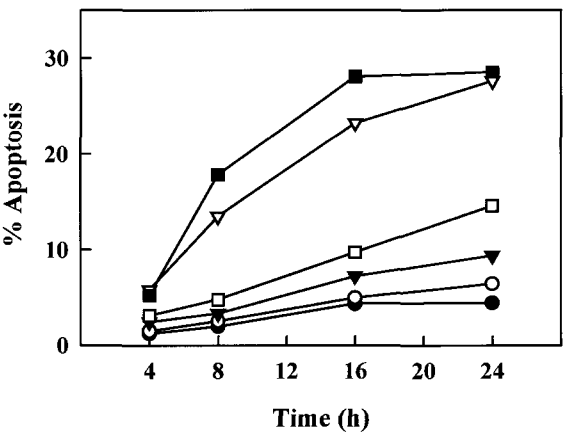

Fig. 3. Kinetics of C_2-ceramide-induced apoptosis in PC12 cells. PC12 cells were treated in the absence (●) or the presence of 5 μM (○), 10 μM (▼), 15 μM (▽), 20 μM (■) and 25 μM (□) C_2-ceramide for the indicated times. Levels of apoptosis were quantitatively determined by Hoechst 33258 staining. Results shown are mean data from at least four separate experiments.

by C_2-ceramide. Experiments were conducted in 35 mm dishes; at the standard seeding density (4×10^4 cells/well) ceramide treatment increased the levels of apoptosis 8-fold (Fig. 4). As the cell plating density increased, the ability of C_2-ceramide (20 μM) to induce apoptosis decreased. Indeed, when cells were seeded at a density of 2×10^5 cells/well the apoptotic effect of exogenous C_2-ceramide disappeared (Fig. 4). These results demonstrate that in PC12 cells apoptosis induced by exogenous ceramide critically depends on cell plating density.

It is becoming increasingly apparent that proteases play an important role during apoptosis. Recent attention has primarily focused on a family of novel cysteine proteases, termed ICE (interleukin-1β converting enzyme)-like proteases, in mediating apoptosis [for reviews see [26,27]]. To determine whether ceramide-induced apoptosis requires ICE-like proteases, we used the irreversible tripeptide inhibitor Z-VAD.FMK. This cell permeable inhibitor blocks apoptosis induced by diverse stimuli in THP.1 cells [28], rat hepatocytes [29] and Jurkat T-cells [30]. Evidence indicates that Z-VAD.FMK inhibits apoptosis by blocking the cleavage of CPP32 [31,32]. Our data demonstrate that Z-VAD.FMK inhibits C_2-ceramide-induced apoptosis, assessed by nuclei staining with Hoechst 33258, in PC12 cells (Table 1); Z-VAD.FMK (50 μM) inhibited ceramide-induced apoptosis by 51%, whereas Z-VAD.FMK (100 μM) completely blocked ceramide-induced nuclear changes. Although Z-VAD.FMK prevented changes in the nuclear morphology, it failed to prevent the cell shrinkage, detachment from the substratum and blebbing of the cell membrane observed in apoptosis. These data establish a role for ICE-like proteases, in particular CPP32, in ceramide-induced apoptosis in PC12 cells and supports recent evidence demonstrating the activation of CPP32 in ceramide-induced apoptosis [33].

In summary, our findings show that exogenous ceramide can specifically induce apoptosis in PC12 cells. The effect of ceramide is time and concentration dependent. Moreover, ceramide-induced apoptosis depends critically on cell plating density, indicating that apoptosis initiated by exogenously added ceramide in PC12 cells is subject to surface dilution kinetics. The failure of bacterial SMase to induce apoptosis suggests that if the generation of endogenous ceramide is important to apoptosis in PC12 cells, the signalling pool of sphingomyelin does not reside in the outer leaflet of the plasma membrane. Finally, our findings establish a role for ICE-like protease activation in ceramide-induced apoptosis in PC12 cells.

Table 1
Z-VAD.FMK prevents C_2-ceramide-induced apoptosis in PC12 cells

	Z-VAD.FMK (μM)		
	−	50	100
Control	3.6 ± 0.7	4.2 ± 0.6	3.7 ± 1.3
C_2-ceramide	37.9 ± 2.1	18.6 ± 2.5	3.1 ± 1.6

PC12 cells were incubated in either the absence or the presence of Z-VAD.FMK (50 μM and 100 μM) for 1 h, then further incubated for 20 h in the absence or the presence of C_2-ceramide (20 μM). Z-VAD.FMK was present throughout the experiment. The percentage of apoptotic cells was determined by nuclei staining with Hoechst 33258. Results are expressed as means ± S.D. from four separate experiments.

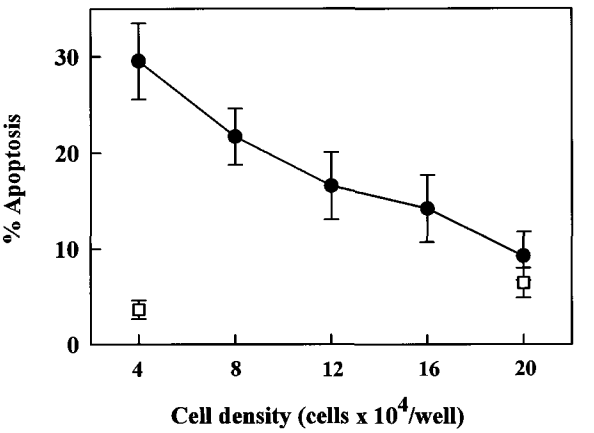

Fig. 4. Ceramide-induced apoptosis depends on cell plating density. PC12 cells were treated in either the absence (□) or the presence of 20 μM C_2-ceramide (●) for 20 h. Levels of apoptosis were determined by Hoechst staining. Results shown are means ± S.D. from four separate experiments.

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