

## The formation of ceramide from sphingomyelin is associated with cellular apoptosis<sup>\*⊙</sup>

Glyn Dawson<sup>⊗</sup>, Rajendra Goswami, John Kilkus, Douglas Wiesner and Sylvia Dawson

*Departments of Pediatrics and Biochemistry and Molecular Biology, University of Chicago, Chicago, U.S.A.*

The apoptotic response of the immature B-cell to the cross-linking of surface IgM receptors provides a good model for cell death and we show in WEHI-231 B-cells that the time course of apoptosis corresponds to the increased formation of ceramide, as measured either by mass (using the diacylglycerol kinase method) or radiolabelling with [<sup>3</sup>H]palmitate. Inhibitors of sphingosine biosynthesis have no effect on cell death induced by anti-IgM in WEHI-231 but inhibitors of ceramidase accelerate apoptosis, suggesting that activation of sphingomyelinase is the key event in apoptosis. We have demonstrated this by *in vitro* assay of neutral sphingomyelinase. Apoptosis is also important in normal brain development and neuronal survival is dependent upon phosphatidylinositol 3-kinase (PI3-kinase) activation by growth factors (insulin, nerve growth factor etc.). Withdrawal of these growth factors or inhibition of PI3-kinase with wortmannin or LY294002 activated the pro-apoptotic CPP32 (Yama/Apopain/caspase 3, EC 3.4.22), activated neutral sphingomyelinase and increased ceramide formation in an immortalized dorsal root ganglion cell line F-11. Protection against apoptosis can be achieved by overexpression of the bcl2 family of proteins or addition of drugs which elevate cAMP levels. cAMP protects against apoptosis induced by either wortmannin or staurosporine. The specificity for cAMP was confirmed by showing protection with the specific agonist (Sp)cAMPS and increased killing with the antagonist (Rp)cAMPS. However, cAMP did not protect against ceramide killing, suggesting that there are at least two major pathways of apoptosis in neuronal cells.

\*Presented at the International Symposium on Structure, Metabolism, and Functions of Glycosphingolipids and Sphingolipids in Warsaw, Poland, September 4-6, 1997.

⊙Supported in part by USPHS Grant NS-36866 and the Brain Research Foundation.

⊗Correspondence to: Dr. Glyn Dawson, Department of Pediatrics, HM 4068, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, U.S.A.; tel: 773 702 6430; fax: 773 702 9234; e-mail: dawg@midway.uchicago.edu

**Abbreviations:** DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCD, programmed cell death (apoptosis); PI3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; Bt<sub>2</sub>cAMP, dibutyryl cyclic AMP; TPA, tetradecanoylphorbol acetate; C2-ceramides, 2-N-acyl sphingosines.

Ceramides (a family of 2-*N*-acyl sphingolipids) appear to be an intracellular signal for the regulation of the cell cycle and a cascade that eventually leads to apoptosis (Kendler & Dawson, 1990; Yanaga & Watson, 1992; Kolesnick & Golde, 1994; Quintans *et al.*, 1994; Hannun, 1994; Chang *et al.*, 1995; Gottschalk *et al.*, 1995; Yao *et al.*, 1995; Verheij *et al.*, 1996; Wiesner & Dawson, 1996a, b, Wiesner *et al.*, 1997). A sphingomyelin cycle has been identified in which the action of extracellular signalling agents (for example cytokines such as TNF- $\alpha$ ) results in the activation of one or more sphingomyelinases, cleavage of membrane sphingomyelin, formation of ceramide and the activation of multiple cellular and biochemical targets (Kolesnick & Golde, 1994; Quintans *et al.*, 1994; Hannun, 1994). Inhibitors of sphingosine biosynthesis have no effect on the programmed cell death but inhibitors of ceramidase accelerate apoptosis (Kolesnick & Golde, 1994; Quintans *et al.*, 1994; Hannun, 1994; Wiesner *et al.*, 1997), suggesting that activation of sphingomyelinase is an early event in apoptosis. The addition of exogenous water-soluble ceramide analogs to cells induces apoptosis and the phenomenon is specific since dihydroceramides are ineffective (Kolesnick & Golde, 1994; Quintans *et al.*, 1994; Hannun, 1994; Wiesner *et al.*, 1997). The endogenous formation of ceramide in response to pro-apoptotic agents is usually associated with a concomitant reduction in sphingomyelin levels and hydrolysis of sphingomyelin to ceramide during apoptosis. This has been demonstrated by *in vitro* assay of neutral sphingomyelinase (Hannun, 1994; Wiesner *et al.*, 1997), although there have been claims that activation of an acidic sphingomyelinase may be also required for certain types of apoptosis (Kronke *et al.*, 1995).

The role of this sphingolipid in apoptosis has suggested that its metabolism is a logical target for attempts to either accelerate apoptosis, and thereby kill cancer cells or protect against apoptosis, and thereby protect neural cells from degeneration. The apoptotic re-

sponse of the immature B-cell to the cross-linking of surface IgM receptors provides a good physiological model for cell death and we have shown in WEHI-231 cells that the time course of apoptosis corresponds to the increased formation of ceramide (Quintans *et al.*, 1994; Gottschalk *et al.*, 1995; Wiesner *et al.*, 1997). The bcl2/bclxL family of proteins are protective against apoptosis (Zhang *et al.*, 1996) and are typically elevated in tumor cells, making them a good knock-out target for efforts to increase apoptosis in tumors. However, there are cellular differences which make them a problematic target, since in WEHI-231 cells, bclxL overexpression protected against C2-ceramide and anti-IgM-induced apoptosis whereas bcl2 did not (Wiesner *et al.*, 1997). For this reason, the ceramidase inhibitor oleoylethanolamine seems to be a better prototype for drugs which might increase apoptosis since it kills a broad spectrum of cells (Wiesner & Dawson, 1996a, b, Wiesner *et al.*, 1997; Mangoura & Dawson, 1997).

Protection against apoptosis is one of the major goals of clinical efforts to slow or reverse degenerative diseases such as Alzheimers, Parkinsons, ALS and multiple sclerosis. Protein kinase C activation is generally protective against apoptosis and one of its relevant actions is to phosphorylate the pro-apoptotic protein BAD and prevent it from inactivating the protective proteins of the bcl family (Ito *et al.*, 1997). We now show that anti-IgM-induced apoptosis in WEHI-231 B-cells can be prevented by co-addition activators of the protein kinase C such as phorbol esters. Conversely, staurosporine, initially described as an inhibitor of protein kinase C (PKC) (Jalava *et al.*, 1993), is able to induce apoptosis in a wide range of cell types. However the mechanism of action of staurosporine is probably more complex than simple PKC inhibition since other inhibitors of PKC such as K252a and KT5720 (Bertrand *et al.*, 1994) do not induce apoptosis. One intriguing action of staurosporine is its ability to activate

a specific 60-kDa serine/threonine kinase (Pavlovic-Surjancev *et al.*, 1993; Mangoura & Dawson, 1997) for up to 24 h. This novel action may explain its ability to induce apoptosis in a range of cells from chondrocytes (Bertrand *et al.*, 1994) to embryonic neurons (Wiesner & Dawson, 1996a) and neuroblastoma cell lines (Wiesner & Dawson, 1996b; Dawson *et al.*, 1997), although there is no direct evidence to support this.

A major signalling pathway involved in protection against apoptosis is the PI 3-kinase pathway, which is activated mainly by growth factors such as insulin and nerve growth factor. Withdrawal of these growth factors induces apoptosis (Kennedy *et al.*, 1997). Both the fungal metabolite wortmannin and the structurally unrelated LY294002 induce both inhibition of PI3-kinase and apoptosis (Arcaro & Wymann, 1993; Straub & Sharp, 1996). A major kinase activated by the lipid product of PI3-kinase (PtdIns3,4P<sub>2</sub>) is protein kinase B (Akt). Several studies have shown that Akt is protective against apoptosis (Burgering & Coffey, 1995; James *et al.*, 1996; Kennedy *et al.*, 1997). Thus cells constitutively expressing a myristoylated (membrane-associated) form of Akt (Myr-Akt) or the oncogenic v-Akt are resistant to wortmannin (Kennedy *et al.*, 1997).

We now report that apoptosis induced by either staurosporine or PI3-kinase inhibitors involves increased formation of ceramide from sphingomyelin, and discuss the ability of other signalling molecules to regulate this pathway.

## MATERIALS AND METHODS

### Materials

Staurosporine and wortmannin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). LY 294002 was from Biomolecular Research Labs Inc. (Plymouth Meeting, PA, U.S.A.), DEVD-AFC was from Biomol. Inc

(P-409), and Rp- and Sp-cAMP were from RBI (Natick, MA, U.S.A.).

### Cell culture

WEHI-231 cells were maintained in suspension culture as described previously (Quintans *et al.*, 1994; Gottschalk *et al.*, 1995). F-11 cells were maintained in 10-cm plates in Dulbecco's modified Eagles medium (DMEM) supplemented 10% fetal calf serum and 1% gentamicin (Francel *et al.*, 1987; Dawson *et al.*, 1997). Wortmannin, staurosporine and LY294002 were added in DMSO, keeping final concentration below 1%.

### Cell viability assay

**A.** For WEHI 231 cells: Percent cell viability was measured by propidium iodide staining followed by fluorescence activated cell sorting analysis (Gottschalk *et al.*, 1995).

**B.** For F-11 cells: The modified MTT assay (Hansen *et al.*, 1989) was done in 24-well culture dishes, each well containing 250  $\mu$ l of media. After treatment, 25  $\mu$ l of stock MTT (5 mg/ml in sterile PBS) was added and incubated for 45 min at 37°C. Finally, a solution of 250  $\mu$ l of 10% SDS in 0.01 M HCl was added. After overnight incubation, the absorption value at 570 nm was determined with a Hitachi spectrophotometer. Viability was determined as % survival = [(exp(-)blank)/(control(-)blank)] where exp is the reading for the treated cells, control is untreated cells, and blank is MTT added just to the media. We have previously demonstrated that MTT results correlate well with cell counts and DNA fragmentation (Wiesner & Dawson, 1996a, b; Wiesner *et al.*, 1997).

### DNA fragmentation assay for apoptosis using Hoescht fluorescence

Cells (10<sup>6</sup>) were lysed in 0.1% Triton X-100, 5 mM Tris, 20 mM EDTA, pH 8.0. After thorough mixing, the lysate was centrifuged at



30000  $\times g$  at 4°C for 40 min. Aliquots (50  $\mu$ l) were added to 2 ml of TNE (100 mM Tris, 10 mM EDTA, 2 M NaCl, pH 7.4) and 0.1 ng/ml Hoechst 33285 dye. DNA lost to the media could be measured in the same way by first adjusting 1 ml of media to 25 mM EDTA and centrifuging at 20000  $\times g$  at 4°C for 40 min. A 200  $\mu$ l aliquot of the supernatant was added to 2 ml TNE and Hoechst 33285 for fluorescence measurement. Fluorescence was measured in a Varian Fluorometer at excitation wavelength of 365 nm and emission wavelength of 460 nm. DNA values were calculated by comparison with a standard curve of calf thymus DNA described previously (Quintans *et al.*, 1994; Wiesner & Dawson, 1996a, b).

**Diacylglycerol kinase assay for identification and quantitation of ceramide.** A modification of the method of Preiss *et al.* (1988) was used. Lipids were isolated in a Folch partition as previously described (Quintans *et al.*, 1994; Wiesner & Dawson, 1996a, b) dried and the ceramide was converted to ceramide 1-[<sup>32</sup>P]phosphate by *E. coli* diacylglycerol kinase. Labeled lipids (ceramide-1-phosphate and phosphatidic acid) were separated by high-performance thin-layer chromatography. Following autoradiography, spots corresponding to ceramide-1-phosphate were scraped and counted in a scintillation counter. Quantitation of ceramide was based on a standard curve of known amounts of ceramide.

**Lipid synthesis and catabolism.** Cells ( $2 \times 10^6$ ) were cultured for 24 h in media containing 10  $\mu$ Ci [<sup>3</sup>H]palmitate in the presence or absence of the indicated reagents (Quintans *et al.*, 1994; Wiesner & Dawson, 1996a, b). Under these conditions, sphingolipids are labelled well and palmitate is broken down to acetate and recycled such that *de novo* synthesized cholesterol is also labelled. Lipids were extracted as described previously (Quintans *et al.*, 1994; Wiesner & Dawson, 1996a, b). The upper phase was discarded and the lower phase evaporated to dryness under nitrogen.

Samples were run on HPTLC as described previously (Quintans *et al.*, 1994; Wiesner &

Dawson, 1996a, b), on a 10 cm  $\times$  10 cm LHP-K TLC plate (Whatman) in chloroform/methanol/glacial acetic acid (94:1:5, by vol.) which resolves cholesterol (Chol) from the ceramide doublet. The upper band (L) consisted mostly of a mixture of C22:0, C24:0 and C24:1 fatty acids, while the lower band (S) consisted primarily of C16:0, C16:1 and C18:0 fatty acids.

Sphingomyelin was resolved from phosphoglycerides in chloroform/methanol/acetic acid/water (65:25:8.8:4.5, by vol.). Plates were sprayed with En3Hance (Dupont) and developed overnight with Xomat-AR film (Kodak) to identify the bands by comparison with authentic standards. Bands were then scraped for liquid scintillation counting and results expressed as either the change in ceramide content (normalized to total cell protein) or as the ratio to sphingomyelin as an index of sphingomyelin hydrolysis.

**Sphingomyelinase assay.** Microsomal and cytosolic cellular fractions were prepared as described previously (Wiesner *et al.*, 1997). Neutral sphingomyelinase activity was assayed with [<sup>14</sup>C-methyl]sphingomyelin (40000 c.p.m. in 1 nmol bovine brain sphingomyelin (Sigma) in 0.2% Triton X-100, 80 mM MgCl<sub>2</sub> and 0.1 M Tris/HCl, pH 7.4, solubilized by sonication and vortexing. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 0.5 ml chloroform/methanol (2:1, v/v). The samples were pulse-vortexed 3 times for 30 s then briefly centrifuged to separate the two phases. The upper phase, containing labelled phosphorylcholine released from sphingomyelin was transferred to scintillation vials and counted by liquid scintillation counting. Negative controls containing no enzyme or boiled cell lysate were run concomitantly.

Acid sphingomyelinase (lysosomal) activity was measured as above except that 0.1 M sodium acetate, pH 5.5, containing 5 mM EDTA replaced Tris/HCl.

### Assay of phosphatidylinositol-3-kinase activity

This activity was measured with a kit from Upstate Biotechnology Inc. using phosphatidylinositol as the substrate, [ $\gamma$ - $^{32}$ P]ATP as the phosphate donor and HPTLC to resolve the polyphosphoinositide products.

### Assay of CPP32 activity

Cells were treated with staurosporine (20 nM), or wortmannin (4 nM) for increasing periods of time, harvested, washed with phosphate-buffered saline and the pellets resuspended in 25 mM Hepes buffer, pH 7.4, containing 2 mM dithiothreitol, 5 mM EDTA and 10 mM digitonin (Xiang *et al.*, 1996). Hydrolysis of the DEVD-AFC substrate was followed for 15 min by fluorometry (excitation 400 nm, emission 505 nm) and activity calculated from the slope.

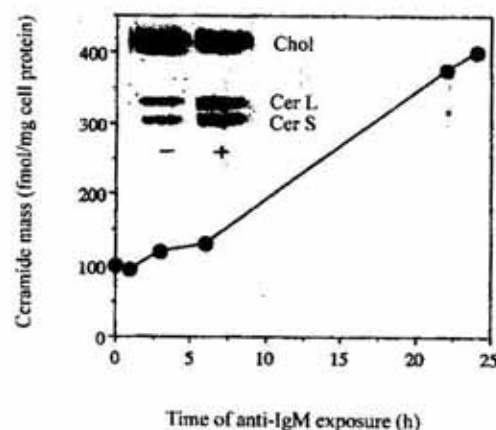
## RESULTS

### Ceramide formation corresponds to the time-course of apoptosis in B-cells

To establish the role of ceramide as a cause of apoptosis in cells undergoing physiological cell death, the formation of ceramide in WEHI-231 cells treated with anti-IgM was measured both by [ $^3$ H]palmitate labelling and by absolute determination of mass by converting ceramide to ceramide-1-[ $^{32}$ P]phosphate with bacterial diacylglycerol kinase (Fig. 1). We have previously reported that apoptosis could be reversible for up to 6 h by removing anti-IgM and it can be seen that there is no increase in ceramide over this initial 6 h period. After 24 h we typically see 60–80% apoptosis (Quintans *et al.*, 1994) and this correlates with the 4-fold increase in ceramide. There was good agreement between the two methods and it can be seen (inset to Fig. 1) that although the ratio of the C22+C24 fatty acid forms to the C16+/C18 forms varies between different

cell lines (Table 1), in WEHI-231 cells it is about 2:1 and both forms increase during apoptosis induced by 24 h exposure to anti-IgM.

Both lipopolysaccharide (1 mg/ml) and phorbol esters (tetradecanoylphorbol ester, 1  $\mu$ M) protected against apoptosis induced by anti-IgM (Fig. 2A) and this is reflected in their ability to block the formation of ceramide from sphingomyelin (Fig. 2B). Fumonisin B1 inhibited sphingolipid synthesis by 50% at 25  $\mu$ M for 24 h (as expected from previous work) but did not affect either apoptosis in B-cells induced by anti-IgM or the formation of ceramide from sphingomyelin (not shown; Wiesner *et al.*, 1997) suggesting that ceramide is not of biosynthetic origin. In contrast, the ceramidase inhibitor, oleoylethanolamine (OE) (25  $\mu$ M), accelerated apoptosis induced by anti-IgM (Fig. 2A) and increased the formation of ceramide from sphingomyelin (Fig. 2B). The addition of bacterial (*Staphylococcus aureus*) sphingomyelinase to WEHI-231 resulted in cell death (Fig. 2A) and an increase in the ceramide:sphingomyelin ratio (Fig. 2B).



**Figure 1.** Formation of ceramide in WEHI-231 cells exposed to anti-IgM for increasing time periods, measured as described in the text.

**Inset:** Autoradiogram of  $^3$ H-labelled lipids separated by HPTLC as described in the text. Chol, cholesterol; Cer L, mixture of C22:0, C24:0 and C24:1 ceramide forms; Cer S, mixture of C16:0 and C18:0 ceramide forms.

### Staurosporine and PI3-kinase inhibitors induce apoptosis and ceramide formation by different mechanisms in the immortalized dorsal root ganglion cell line F-11

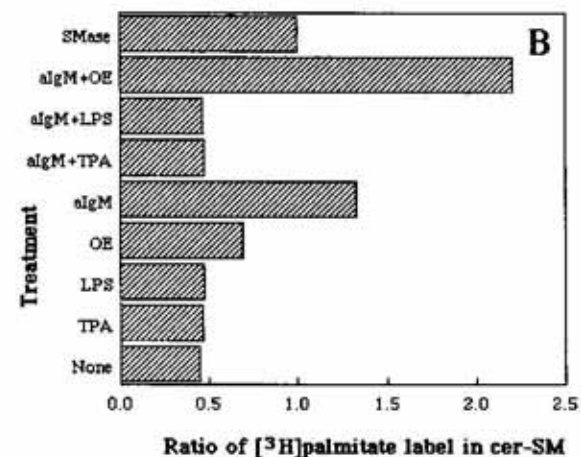
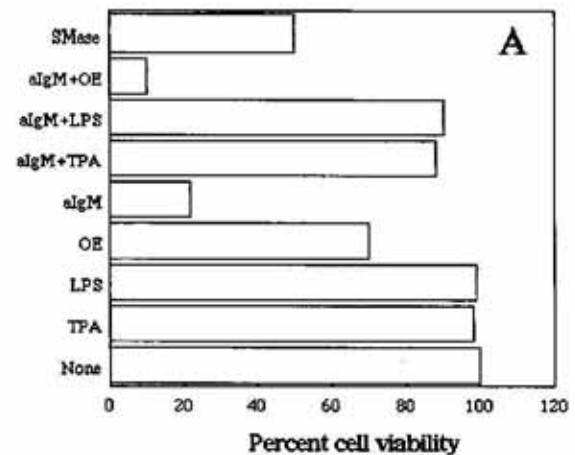
Both staurosporine and the PI3-kinase inhibitors wortmannin and LY294002 induced ceramide formation (Fig. 3A), decreased cell viability in 24 h as judged by the MTT assay (Fig. 3B) and induced DNA fragmentation (Fig. 3C). Thus the results were comparable to the role of ceramide in apoptosis as observed in the B-cell line WEHI-231.

The mechanism of action of staurosporine and wortmannin is different since staurosporine had no effect on PI3-kinase activity in F-11 cells whereas wortmannin was a potent inhibitor of this activity (Fig. 4). Despite this difference, both wortmannin and staurosporine activated CPP32 (Yama/Apopain/caspase-3) the protease required to initiate the degradation phase of apoptosis (Kuida *et al.*, 1996), in a time-dependent manner (Fig. 5). This suggests a convergence of the killing pathways prior to CPP32. Surprisingly, exogenous C2-ceramide at concentrations (25  $\mu$ M), which induced 50% apoptosis of F-11 cells, did not activate CPP32 under these conditions (not shown).

After 24 h of treatment with wortmannin (4  $\mu$ M) we could show by *in vitro* assay that neutral sphingomyelinase activity (pH 7+Mg<sup>2+</sup>) was increased 2-fold whereas acid sphingomyelinase activity (pH 5) was unaffected (Fig. 6). We observed a similar activation after treatment with staurosporine (50 nM) (not shown).

### Cyclic AMP protects against staurosporine-induced apoptosis but not against ceramide-induced apoptosis

When F-11 cells were pretreated with Bt<sub>2</sub>cAMP for up to 36 h prior to addition of either 20 or 50 nM staurosporine for a further 24 h to induce apoptosis, we observed a protective effect. Thus an 18 h pretreatment gave the maximum protection, reducing cell



death by 50 nM staurosporine from > 50% to < 80% over the 24 h treatment period (Fig. 7). The specificity for cAMP was confirmed by showing protection with the specific agonist (Sp)cAMPS (80  $\mu$ M) and increased killing with the antagonist (Rp)cAMPS (80  $\mu$ M) (not shown). Similar protection was observed when the apoptosis was induced by PI3-kinase inhibitors wortmannin and LY294002. However, Bt<sub>2</sub>cAMP was not able to protect against killing by exogenous C2-ceramide (25  $\mu$ M) as shown in Fig. 8.

### DISCUSSION

The normal program of development in vertebrates results in the controlled death (apoptosis) of many cells and there is increasing evidence for a key role for sphingolipid metabolites, especially ceramide in this process. The

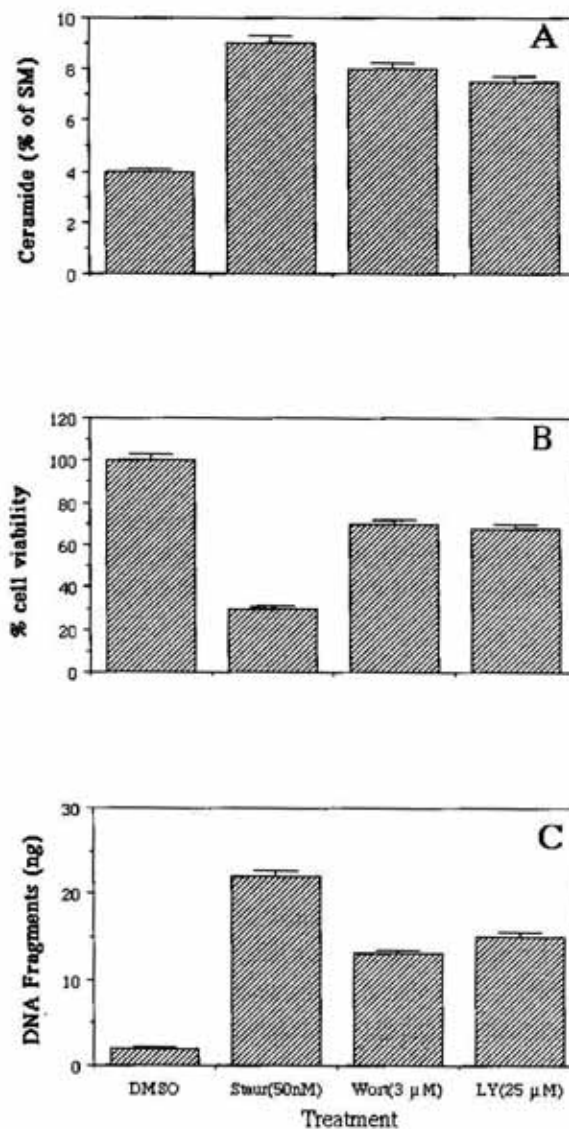


**Table 1. Different ratios of fatty acylated forms of ceramide in different cell lines.**

Cultured cells were labelled with [ $^3\text{H}$ ]palmitate for 24 h, lipids extracted and ceramide isolated by HPTLC as described in the text. The plate was exposed to film and the ratio of ceramide bands quantitated by densitometry.

Cell-line	Ceramide
	$C_{22} + C_{24} / C_{16} + C_{18}$ fatty acids ratio
B-Cells	
WEHI-231	1.5
RAMOS	2.0
T-cells	
JURKAT	0.25
PD388 Macrophage	4.0
Human skin fibroblasts	0.2
F-11	0.35

role of apoptosis in normal brain development can be dramatically illustrated by deleting one of the key caspases in the apoptotic process (CPP32) by homologous recombination in a mouse (Kuida *et al.*, 1996). In these CPP32 knock-out mice, brain development is profoundly affected because neurons do not die at the appropriate time. Although the evidence for a role for ceramide in cell death is quite strong (Kendler & Dawson, 1990; Kolesnick & Golde, 1994; Quintans *et al.*, 1994; Hannun, 1994; Chang *et al.*, 1995; Gottschalk *et al.*, 1995; Verheij *et al.*, 1996; Wiesner & Dawson, 1996a, b; Wiesner *et al.*, 1997), the subcellular localization of ceramide must be extremely important since all healthy cells contain some ceramide. The working hypothesis is that sphingomyelin on the outer surface of the plasma membrane is hydrolysed by a neutral sphingomyelinase and that the ceramide generated is translocated to the inner leaflet of the bilayer where it acts to recruit proteins. Thus ceramide action is envisioned as analogous to the way in which diacylglycerols activate protein kinase C or PtdIns3,4P<sub>2</sub> activates protein kinase B (Akt). What are these down-



**Figure 3. Staurosporine and PI3-kinase inhibitor (wortmannin and LY294002) induced ceramide formation (panel A), cell viability as measured by the MTT assay (panel B) and induced DNA fragmentation (panel C), following 24 h of treatment of F-11 cells at the concentrations indicated.**

Experimental details are given in the text. Results are the mean of 3 experiments  $\pm$  S.E.

stream targets? One report (Smyth *et al.*, 1996) suggests that ceramide can directly activate one member of the caspase family (pro-ICE or caspase 1) but other kinase/phosphatase targets for ceramide have been claimed (e.g., Westwick *et al.*, 1995) and the mechanism by which increased levels of ceramide induce apoptosis remain to be resolved. Not all

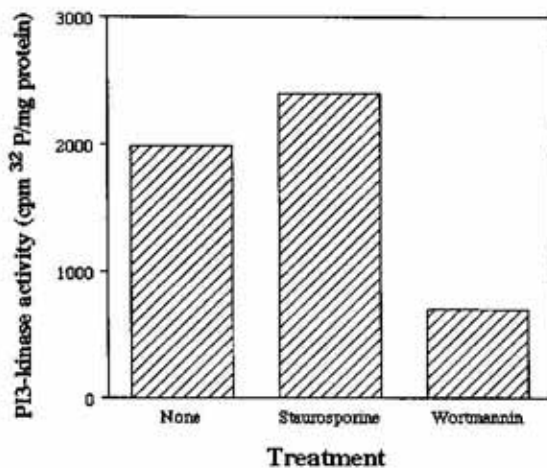


Figure 4. PI3-kinase activity following treatment of F-11 cells with staurosporine (50 nM) or wortmannin (4  $\mu$ M) for 24 h as described in the text.

apoptosis may require ceramide and Susin *et al.* (1997) have attempted to explain why the anti-apoptotic protein bcl<sub>2</sub> prevents both ceramide- and prooxidant-induced apoptosis yet fails to prevent Fas-triggered apoptosis in most cell types. They proposed that Fas-induced apoptosis could proceed *via* ICE, mitochondrial permeability transition, and CPP32 in a bcl-2-independent manner or *via* ceramide-activated pathways which are regulated by bcl-2 but do not necessarily require CPP32. This may not hold true for all cell types but suggests an explanation for the different results obtained from studies in different laboratories with different cell types.

Although we know that growth factors and the resultant phosphorylation of specific tyrosine residues protect cells against apoptosis we know very little of the mechanism by which other signalling molecules affect the process of apoptosis. However, it seems clear from previous work and the work presented here that ceramide is involved in apoptosis and that these agents must affect either upstream regulators of sphingomyelinase or the downstream targets of ceramide. We therefore investigated the two major kinase signalling pathways in the cell, protein kinase C and protein kinase A. The activation of PKC and the resultant phosphorylation of specific serine

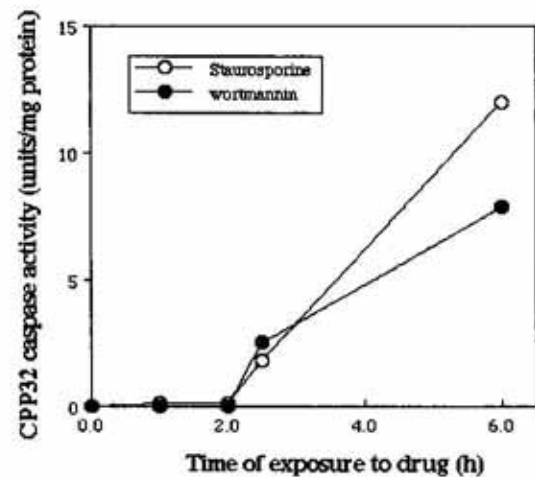


Figure 5. Time-course of induction of CPP32 protease activity in F-11 cells exposed to staurosporine (○) (50 nM) or wortmannin (●) (4  $\mu$ M) for increasing periods of time.

residues has been shown to be anti-apoptotic in some cell systems. However in others, ceramide has been claimed to actually activate a specific isoform of PKC (Muller *et al.*, 1995). In WEHI-231 we found activators of PKC (phorbol esters, tetradecanoylphorbol acetate, TPA) to be protective against anti-IgM-induced apoptosis whereas in F-11 cells we found TPA to be ineffective against staurosporine, ceramide or wortmannin-induced apoptosis (not shown).

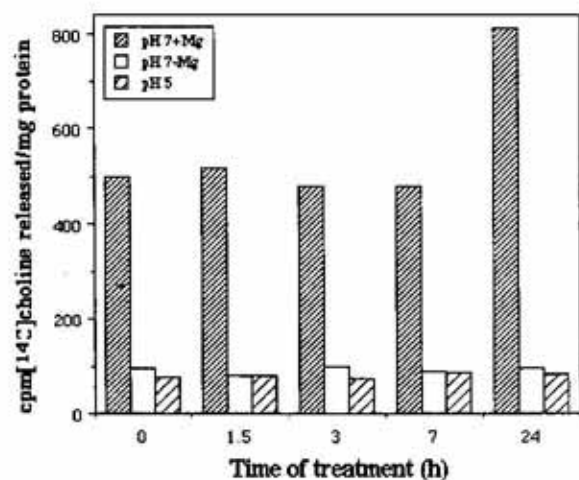


Figure 6. *In vitro* assay for sphingomyelinase in F-11 cells treated with wortmannin (4  $\mu$ M) for increasing periods of time. Experimental details are given in the text.



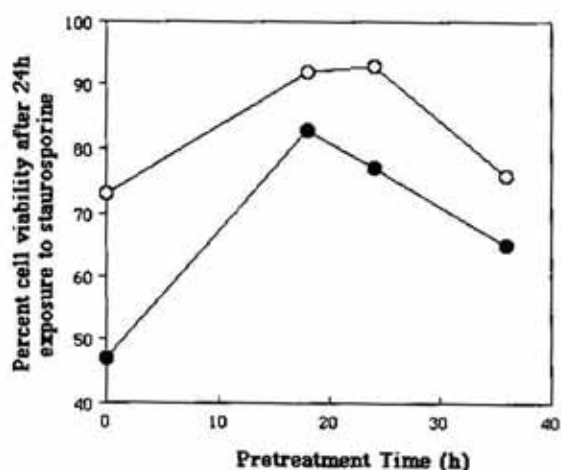


Figure 7. Increasing cell viability (measured by MTT assay) of F-11 cells following pretreatment with  $Bt_2cAMP$  for increasing periods of time, before exposure to 20 nM staurosporine (○) or 50 nM staurosporine (●) for 24 h.

Experimental details are given in the text.

In this report we present more evidence for a protective role for protein kinase A. Thus the addition of  $Bt_2cAMP$  or a cAMP agonist such as (Sp)cAMPS (Yusta *et al.*, 1988) protected F-11 cells against apoptosis. The ability of cAMP to protect against apoptosis has been observed in a number of other cells, for example cardiac myocytes (Wu *et al.*, 1997). A likely mechanism involves protein kinase A since there are reports (Kauffmann-Zeh *et al.*, 1997; Zhang *et al.*, 1995) that PKA-mediated inhibition of Raf-1 kinase is protective against apoptosis. In support of this we show that apoptosis can be exacerbated by adding a cAMP antagonist such as (Rp)cAMPS. However, such a model is obviously too simple since it does not take into account the possible role of  $\beta\gamma$ -subunits which are part of the receptor-G-protein-adenylate cyclase complex but can also activate PI3-kinase  $\gamma$  (Stoyanova *et al.*, 1997) and help protect the cell against apoptosis. The freeing of  $\beta\gamma$ -subunits following increased cAMP could activate PI3-kinase and help protect cells against apoptosis.

Our working hypothesis is that agonist-mediated increases in cAMP may act as a regulator of the apoptotic process and that

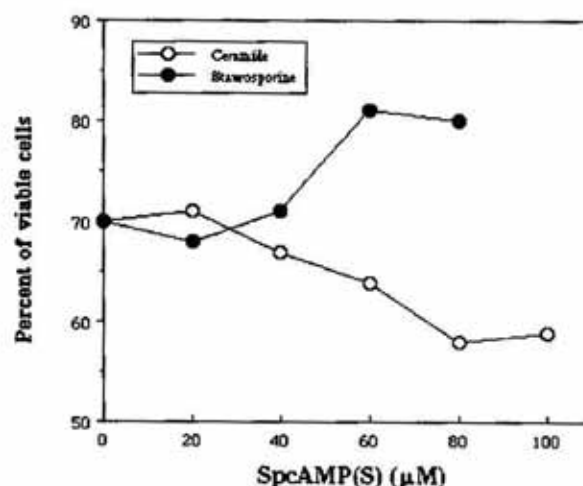


Figure 8. Effect of cAMP analog (Sp)cAMPs on the ceramide (●) and staurosporine (○) induced apoptosis of F-11 cells, killing was assayed by MTT test.

Experimental details are given in the text.

these effects are upstream of ceramide since  $Bt_2cAMP$  did not protect against ceramide-induced apoptosis. However, the precise understanding of the role of sphingolipid metabolism in apoptosis must await the cloning of the neutral sphingomyelinase (and the defining of its regulatory elements) and the clearer definition of the downstream targets of ceramide.

## REFERENCES

- Arcaro, A. & Wymann, M.P. (1993) The fungal alkaloid wortmannin inhibits phosphatidylinositol 3-kinase. *Biochem. J.* **296**, 297-301.
- Bertrand, R., Solary, E., O'Conner, P., Kohn, K.W. & Pommier, Y. (1994) Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res.* **211**, 314-321.
- Burgering, B.M. & Coffey, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **373**, 574-578.
- Chang, Y., Abe, A. & Shayman, J.A. (1995) Ceramide formation during heat shock: A potential

- mediator of  $\alpha$ B crystallin transcription. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12275-12279.
- Dawson, G., Dawson, S.A. & Goswami, R. (1997) Chronic exposure to kappa opioids enhances the susceptibility of immortalized neurons (F-11k7) to apoptosis-inducing drugs by a mechanism that involves ceramide. *J. Neurochem.* **68**, 2363-2370.
- Francel, P.C., Harris, K., Smith, M., Fishman, M.C., Dawson, G. & Miller, R.J. (1987) Neurochemical characteristics of a dorsal root ganglion X neurotumor hybrid cell line. *J. Neurochem.* **48**, 1624-1631.
- Gottschalk, A., McShan, C., Kilkus, J., Dawson, G. & Quintans, J. (1995) Resistance to anti-IgM-induced apoptosis in a WEHI-231 subline is due to insufficient production of ceramide. *Eur. J. Immunol.* **25**, 1032-1038.
- Hannun, Y.A. (1994) The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* **269**, 3125-3128.
- Hansen, M., Nielsen, S. & Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Meth.* **119**, 203-210.
- Ito, T., Deng, X., Carr, B. & May, S. (1997) Bcl-2 Phosphorylation required for anti-apoptosis function. *J. Biol. Chem.* **272**, 11671-11673.
- Jalava, A., Akerman, K. & Heikkila, J. (1993) Protein kinase inhibitor, staurosporine, induces a mature neuronal phenotype in SH-SY5Y human neuroblastoma cells through an alpha-, beta-, and zeta-protein kinase C-independent pathway. *J. Cell Physiol.* **155**, 301-312.
- James, S.R., Downes, C.P., Gigg, R., Grove, S.J.A., Holmes, A.B. & Alessi, D.R. (1996) Specific binding of the akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem. J.* **315**, 709-713.
- Kauffmann-Zeh, A., Rodriguez-Viciano, P., Ulrich, E., Gibert, Ch., Coffey, P., Downwards, J. & Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-546.
- Kendler, A. & Dawson, G. (1990) Progressive hypoxia inhibits the *de novo* synthesis of galactosylceramide in cultured oligodendrocytes. *J. Biol. Chem.* **265**, 12259-12266.
- Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tschlis, P.N. & Hay, N. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes & Develop.* **11**, 701-713.
- Kolesnick, R. & Golde, D. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signalling. *Cell* **77**, 325-328.
- Kuida, K., Zheng, T., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. & Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**, 368-370.
- Mangoura, D. & Dawson, G. (1997) Programmed cell death in cortical chick embryo astrocytes is associated with activation of protein kinase PK60 and ceramide formation. *J. Neurochem.* (in press).
- Muller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D. & Pfizenmaier, K. (1995) PKC  $\zeta$  is a molecular switch in signal transduction of TNF- $\alpha$ , bifunctionally regulated by ceramide and arachidonic acid. *EMBO J.* **14**, 1961-1969.
- Pavlovic-Surjancev, B., Cahill, A.L. & Perlman, R.L. (1993) Staurosporine activates a 60000  $M_r$  protein kinase in bovine chromaffin cells that phosphorylates myelin basic protein *in vitro*. *J. Neurochem.* **61**, 697-703.
- Quintans, J., Kilkus, J., McShan, J.L., Gottschalk, A.R. & Dawson, G. (1994) Ceramide mediates the apoptotic response of WEHI cells to anti-immunoglobulin, corticosteroids and irradiation. *Biochem. Biophys. Res. Commun.* **202**, 710-714.
- Schutze, S., Potthoff, T., Machleidt, D., Berkovic, K., Wiegmann, H. & Kronke, M. (1992) TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell* **71**, 765-776.
- Smyth, M.J., Perry, D.K., Zhang, J., Poirier, G.G., Hannun, Y.A. & Obeid, L.M. (1996) prICE: A downstream target for ceramide-induced apoptosis and for the inhibitory action of Bcl-2. *Biochem. J.* **316**, 25-28.

- Susin, S.A., Zamzami, N., Castedo, M., Daugas, E., Wang, H.-G., Geley, S., Fassy, F., Reed, J.C. & Kroemer, G. (1997) The central executioner of apoptosis: Multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. *J. Exp. Med.* **186**, 25-37.
- Straub, S.G. & Sharp, G.W.G. (1996) A wortmannin-sensitive signal transduction pathway is involved in the stimulation of insulin release by vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide. *J. Biol. Chem.* **271**, 1660-1668.
- Stoyanova, S., Bulgarelli-Leva, G., Kirsch, C., Hanck, T., Klinger, R., Wetzker, R. & Wyman, M.P. (1997) Lipid kinase and protein kinase activities of G-protein-coupled phosphoinositide 3-kinase  $\gamma$ : structure-activity analysis and interactions with wortmannin. *Biochem. J.* **324**, 489-495.
- Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Dyriakis, J.M., Halmovity-Friedman, A., Fuks, Z. & Kolesnick, R.N. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* **380**, 75-79.
- Westwick, J.K., Bielawska, A.E., Dbaibos, G., Hannun, Y.A. & Brenner, D.A. (1995). Ceramide activates the stress-activated protein kinases. *J. Biol. Chem.* **270**, 22689-22692.
- Wiesner, D.A. & Dawson, G. (1996a) Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway. *J. Neurochem.* **66**, 1418-1425.
- Wiesner, D.A. & Dawson, G. (1996b) Programmed cell death in neurotumor cells involves the generation of ceramide. *Glycoconjugate J.* **13**, 327-333.
- Wiesner, D.A., Kilkus, J.P., Gottschalk, A.R., Quintans, J. & Dawson, G. (1997) Anti-immunoglobulin-induced apoptosis in WEHI-231 cells involves the slow formation of ceramide from sphingomyelin and is blocked by bcl-xL. *J. Biol. Chem.* **272**, 9868-9876.
- Wu, C.-F., Bishopric, N.H. & Pratt, R.E. (1997) Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes. *J. Biol. Chem.* **272**, 14860-14866.
- Xiang, J., Chao, D.T. & Korsmeyer, S.J. (1996) Bax-induced cell death may not require interleukin 1 beta-converting enzyme like proteases. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14559-14563.
- Yanaga, F. & Watson, S. (1992) Tumor necrosis factor- $\alpha$  stimulates sphingomyelinase through the 55 kDa receptor in HL-60 cells. *FEBS Lett.* **314**, 297-300.
- Yusta, B., Ortiz-Caro, J., Pascual, A. & Aranda, A. (1988) Comparison of the effects of forskolin and dibutyryl cyclic AMP in neuroblastoma cells: Evidence that some of the actions of dibutyryl cyclic AMP are mediated by butyrate. *J. Neurochem.* **51**, 1808-1817.
- Zhang, J., Alter, N., Reed, J.C., Borner, C., Obeid, L.M. & Hannun, Y.A. (1996) Bcl-2 interrupts the ceramide-mediated pathway of cell death. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5325-5328.
- Zhang, Jin., Zhang, Jun, Benovic, J.L., Sugai, M., Wetzker, R., Gout, I. & Rittenhouse, S.E. (1995) Sequestration of a G-protein  $\beta\gamma$  subunit or ADP-ribosylation of Rho can inhibit thrombin-induced activation of platelet phosphoinositide 3-kinases. *J. Biol. Chem.* **270**, 6589-6594.